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*Erratum*

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Haydak, M. H., L. S. Palmer, M. C. Tanquary and A. E. Vivino

Table 1, page 584:

| Vitamin | MEAN mg. | S.D. mg. | C.V. % |
|---|---|---|---|
| Nicotinic acid | .325 ± 0.045 | 0.274 | 84.3 |

Table 2, page 585: nicotinic acid values should be divided by 100 for all honeys.

# THE MOBILIZATION BY ALCOHOLS OF VITAMIN A FROM ITS STORES IN THE TISSUES

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## INTRODUCTION

The experiments to be reported in this paper are concerned with the increase in concentration of vitamin A of the serum of dogs following the administration of various alcohols. The experimental conditions were so arranged that the increase was not due to increased absorption of the vitamin from the intestinal tract. We shall attempt to show that the increase was due to liberation of the vitamin from stores in the tissues.

The experiments were undertaken to elucidate the manner in which vitamin A may be liberated from its stores in normal and in pathological conditions. Clausen and McCoord ('38) had observed that the concentration of vitamin A of the plasma increases above the normal level several days after the fall in temperature in scarlet fever and in pneumonia. A similar rise in vitamin A concentration of the plasma was observed 1 or 2 days after artificial hyperthermia; this rise may, of course, be due to a decreased rate of deposition in the stores of vitamin A absorbed from the gastrointestinal tract. During convalescence from acute infectious jaundice an even greater temporary rise in level of plasma vitamin A is observed; this rise can most readily be ascribed to liver damage, which results in failure of rapid storage of vitamin A absorbed from the gastrointestinal tract into the blood or lymph, and not to liberation of the vita-

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min from its stores. Somewhat more definite evidence for liberation of vitamin A from tissue stores was found in two children dying of chronic nephritis: Unusually high concentrations were found in the plasma, and very low values were found in the liver. In experiments with rats (which had received a single massive dose of vitamin A and thereafter had received a vitamin A free diet), McCoord ('39) observed that those animals which succumbed to infection with B-paratyphosus muris, had much more vitamin A in the adrenals than did controls; whereas, the vitamin A content of the liver and lung had decreased in the infected animals. After rats had received large daily doses of thyroxin for several weeks the vitamin A content of the livers decreased, while that of the adrenals increased.

Solyanikova ('38) claimed that doses of vitamin D produced first an increase in the vitamin A content of the blood of rats and a decrease in the vitamin A content of the liver and suprarenal capsule. Later, the vitamin A content of the blood and liver decreased while that of the suprarenal capsule increased.

Young and Wald ('40) showed that, in rabbits, the concentration of vitamin A in the blood was increased by the extirpation of a lobe of the liver, by electrical stimulation of the splanchnic nerve, and by the intravenous injection of adrenaline.

Recently Clausen et al. ('40 a) showed that vitamin A could be mobilized from its stores in the tissues by ethyl alcohol. Previously, attempts were made without marked success to mobilize vitamin A by various drugs and experimental procedures. We have studied the effect of injection of adrenaline, histamine, insulin, mecholyl chloride, exsanguination, inhalations of chloroform and of ether, and operative trauma. Repeated bleeding of rabbits gave some evidence of mobilization of vitamin A, for, although considerable amounts of blood were withdrawn, the total quantity of the vitamin in the circulating blood remained almost constant. Prolonged ether anesthesia at times produced a slight rise in the vitamin A concentration of the serum. However, none of these agents

produced an effect comparable to that of ethyl alcohol. Using dogs as subjects, these authors showed that there was a prompt increase in the vitamin A concentration of the serum following the administration of the alcohol.

Pett has devised a method for measuring the rate of visual adaptation of man to dim light. He reported ('40) that this rate was unusually rapid on the day following the ingestion of alcohol, and ascribed the phenomenon to liberation of vitamin A from its stores. Clausen and his associates ('41) then showed that there was an increase in the vitamin A concentration of the serum of man following the ingestion of ethyl alcohol.

On the other hand, Colson ('40) found that tests given soon after the consumption of alcohol by normal individuals showed no significant change in visual acuity, visual fields, color vision and dark adaptation.

In the present paper we report the results of studies of the effects of various alcohols upon the mobilization of vitamin A from its stores in the tissues of the dog.

## METHODS

The methods for the extraction of vitamin A from serum and tissues are described in detail elsewhere (McCoord and Luce-Clausen, '34; Clausen and McCoord, '38).

The vitamin A content of the petroleum ether extracts was determined by means of the Evelyn photoelectric colorimeter according to the technic first suggested by May (Dann and Evelyn, '38), except that the residue containing the vitamin A was dissolved in 0.5 ml. of chloroform, and 10 ml. of the antimony trichloride reagent was added. For the sake of brevity, we shall use the abbreviation E. P. U. for the Evelyn Photoelectric Unit of vitamin A. One E. P. U. is equal to about 3 International Units of vitamin A. The method for the determination of the free and esterified forms of vitamin A has been described briefly (Clausen et al., '40 b); a more detailed report will be published later.

*The vitamin A concentration of the serum in normal dogs
following the administration of various alcohols*

Twelve dogs were used as subjects in this study, a total of twenty-three tests being carried out. The animals were maintained on a diet of commercial dog food [1] with the addition of a small piece of lean meat each day. In all tests, the alcohol was administered in a single dose. In eleven tests the dogs received ethyl alcohol by stomach tube as a 20% aqueous solution by volume of 95% ethyl alcohol, and in five tests by vein as a 30% solution by volume of 95% ethyl alcohol in isotonic salt solution. Three subjects received methyl alcohol by vein as a 30% solution by volume in isotonic salt solution. By vein dog 38-309 received 10 ml. of propyl alcohol diluted with 10 ml. of the salt solution; dog 39-11, 10 ml. of isopropyl alcohol diluted with 20 ml. of the salt solution; and dog 39-191, 5 ml. of butyl alcohol diluted with 65 ml. of the salt solution. The protocols of six of the experiments are given in table 1. In all the dogs of our series, there was a prompt increase in the vitamin A concentration of the serum following the administration of the alcohol except in the case of the animal which received only 5 ml. of butyl alcohol. The amounts of alcohol used in these experiments varied between 5 and 60 ml. Six of the subjects were given more than one test, but the effect of the alcohol did not appear to diminish with a repetition of the test.

While the dogs receiving the larger doses of ethyl alcohol were very drowsy for some hours, neither ethyl nor methyl alcohol appeared to be injurious to the animals in the doses given. Propyl, isopropyl and butyl alcohols were given by vein in doses of 5 to 10 ml. well diluted with the salt solution; they appeared to be quite intoxicating. The dogs barked, struggled and seemed much excited and then became very drowsy. Their blood showed marked hemolysis and in one case hemoglobinuria was observed. However, the animals seemed to recover rapidly.

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[1] Purina Dog Chow.

The coefficient of correlation between the number of milliliters of 95% ethyl alcohol administered and the greatest increase in Evelyn Photoelectric Units of vitamin A per 100 ml. of serum obtained within the 48 hours was 0.447.

We have found that the diet used in the experiments caused little variation in vitamin A concentration of the serum of dogs during the day, or even in the course of several days. To rule out the effect of the absorption of vitamin A from the gastrointestinal tract, food was withheld from the dogs from 18 to 48 hours previous to and during, the experiments.

Biopsies of the liver were obtained in nine of the dogs receiving 95% ethyl alcohol. The coefficient of correlation between the concentration of vitamin A per 100 gm. of liver and the greatest increase in Evelyn Photoelectric Units of vitamin A per 100 ml. of serum obtained within the 48 hours was 0.280.

TABLE 1

*The vitamin A concentration of serum in normal dogs following the administration of various alcohols.*

| *Dog:* | | | | | | |
|---|---|---|---|---|---|---|
| No. | 39–11 | 38–314 | 38–309 | 38–309 | 39–11 | 39–191 |
| Body weight (kg.) | 17 | 18 | 21 | 21 | 17 | 16 |
| *Treatment:* | | | | | | |
| Alcohol | Ethyl 95% | Ethyl 95% | Methyl | Propyl | Isopyl | Butyl |
| Amount | 60 ml. | 20 ml. | 20 ml. | 10 ml. | 10 ml. | 5 ml. |
| How given | Stomach tube | Vein | Vein | Vein | Vein | Vein |
| *Hours of fasting before test:* | 48 | 18 | 24 | 24 | 24 | 24 |
| *Serum: Vit. A per 100 ml. (E.P.U.[1]):* | | | | | | |
| Before test | 569 | 416 | 638 | 488 | 202 | 140 |
| 1½ hours after test | ... | ... | 702 | 781 | 255 | 140 |
| 3 hours after test | 1323 | ... | 1172 | 868 | 255 | 103 |
| 6 hours after test | ... | 862 | .... | 868 | ... | 129 |
| 24 hours after test | 914 | 757 | 2230 | 908 | 206 | ... |
| 48 hours after test | 859 | ... | 2041 | 830 | ... | ... |
| *Liver: Vit. A per 100 gm. (E.P.U.[1]):* | 8,134 | 27,196 | 106,314 | 106,314 | 8,134 | 5,975 |

[1] Evelyn photoelectric unit.

However, the amount of ethyl alcohol given varied between 16 and 60 ml. It is probable that if all the dogs had received an amount of alcohol proportional to their body weights, a better correlation would have been obtained. In two dogs with fistulas of the thoracic duct, the vitamin A concentration of the lymph did not increase after the administration of the alcohol, but a rise in the vitamin A of the serum did occur.

To ascertain from what tissues of the dog vitamin A could be mobilized, three normal animals were sacrificed and the vitamin A concentration and content of twenty-four tissues of each dog were determined. The detailed results of these analyses are to be published in another communication. It was found that vitamin A is widely distributed in the tissues of the dog, both the concentration and total amount of the vitamin being relatively great in the liver, the kidneys, the retroperitoneal and the subcutaneous fat. The high vitamin A content of the kidney may be related to the fact that the vitamin was frequently found in the urine of normal dogs. On the other hand, the gastrointestinal tract, with its contents, contained a very small amount of vitamin A, and can therefore be definitely eliminated as the source of the mobilized vitamin.

Attempts were made to show that the vitamin was liberated directly from the liver, by injecting the alcohol into the portal vein and then analyzing simultaneously the blood from the hepatic vein and the leg vein for some hours thereafter. The results were rather conflicting, so that no definite conclusions could be established.

The administration of ethyl acetate, acetone, paraldehyde, acetaldehyde, acetic acid and hydrochloric acid was not followed by a rise in the vitamin A concentration of the serum.

### The effect of the daily administration of ethyl alcohol

Daily injections of ethyl alcohol were given to three normal dogs, the procedure being as follows: Each morning for 8 days blood specimens were taken, and the dogs then received by vein, 20 ml. of 95% ethyl alcohol diluted with 40 ml. of sterile isotonic salt solution. In the afternoon at about four o'clock,

blood specimens were again obtained and the dogs were given the usual diet. Each dose of alcohol was usually followed by a rise in the vitamin A concentration of the serum. Table 2 gives the protocol of one of these dogs, no. 38-314. However, by the ninth day the tissues surrounding the veins of the animals were so edematous that it was decided to inject the alcohol into the peritoneal cavity. At the same time the dose

TABLE 2

*Effect of the daily administration of ethyl alcohol on the vitamin A concentration of the serum of a normal dog.*

| DATE | ALCOHOL | TIME | E P U [1] VITAMIN A PER 100 ML. SERUM | TIME | E P U VITAMIN A PER 100 ML. SERUM |
|---|---|---|---|---|---|
| | *ml* | A M. | | P M | |
| 3–18–40 | 20 — by vein | 10 | 416 | 4 | 862 |
| 3–19–40 | 20 — by vein | 10 | 757 | 7 | 1055 |
| 3–20–40 | 20 — by vein | 10 | 694 | 4 | 1080 |
| 3–21–40 | 20 — by vein | 10 | 932 | 4 | 1068 |
| 3–22–40 | 20 — by vein | 9 | 872 | 4 | 1367 |
| 3–23–40 | 20 — by vein | 9 | 951 | 4 | 1232 |
| 3–24–40 | 20 — by vein | 9 | 1131 | 4 | 1244 |
| 3–25–40 | 20 — by vein | 9 | 958 | 4 | 1203 |
| 3–26–40 | 40 — intraperitoneal | 9 | 818 | 4 | 1491 |
| 3–27–40 | 40 — intraperitoneal | 9 | 1046 | 4 | 1128 |
| 3–28–40 | 20 — intraperitoneal | 9 | 923 | 4 | 327 |
| 3–29–40 | 20 — intraperitoneal | 9 | 1818 | 4 | 1528 |
| 3–30–40 | 20 — intraperitoneal | 9 | 1147 | 4 | 1115 |
| 3–31–40 | 20 — intraperitoneal | 12 noon | 903 | 6 | 1188 |
| 4–1–40 | 20 — intraperitoneal | 9 | 942 | 2 | 1199 |

[1] Evelyn photoelectric unit.

was increased to 40 ml. of alcohol diluted with 80 ml. of isotonic salt solution. This amount of alcohol proved to be too much, for the dogs slept all of the time and refused to eat. The former dose of 20 ml. of alcohol and 40 ml. of the salt solution was therefore resumed and administered for 5 days. The alcohol was as effective in mobilizing the vitamin A when injected into the peritoneal cavity as when injected by vein. Each dog received fifteen daily injections, a total of 340 ml. of 95% alcohol.

Biopsies of the livers of these dogs and of a control dog were obtained at the beginning and at the end of this experiment. During this period, the concentration of vitamin A in the liver of the control dog, no. 38-272, increased from 31,630 to 35,575 E. P. U. per 100 gm. of tissue, while that of experimental dog no. 38-314 increased from 27,196 to 29,921 units. On the other hand, the concentrations of vitamin A in the livers of the experimental dogs, no. 39-11 and 39-219, decreased from 8,134 to 2,791 and from 1,619 to 974 units per 100 gm., respectively. Unfortunately, we have no accurate record of the intake of vitamin A by the dogs during the period. These changes were not due to differences in the moisture content of the tissues analyzed. Nor is it probable that they were due to errors in sampling, since we have found that specimens taken at the same time from different parts of the periphery of the liver have approximately the same vitamin A concentration. Microscopic sections of the biopsy specimens of these livers were also prepared. Dr. W. B. Hawkins of the Department of Pathology, who very kindly examined the sections, found that there was no significant alteration in the cells of the livers after daily injections of ethyl alcohol.

The urine of these dogs contained vitamin A, but the quantity present was not increased by the administration of the alcohol.

### The effect of alcohol on the absorption of vitamin A in dogs

When normal dogs receive a large dose of vitamin A by mouth, there is a marked increase in the vitamin A concentration of their serum, the highest concentration usually being reached within 12 hours. It seemed important to determine whether alcohol, administered at the same time, would influence this effect. Three normal dogs received in a capsule by mouth 1800 E. P. U. of vitamin A per kilogram of body weight after a fasting blood specimen had been taken. The vitamin A concentration of the serum was determined at intervals during the next 30 hours. After a few days had elapsed, the dogs received by stomach tube 40 ml. of 95% ethyl alcohol di-

luted with isotonic salt solution, and the rise of vitamin A in their serum was determined as in the preceding experiments. A few days later the tests were repeated, the vitamin A and alcohol being administered at the same time.

The results obtained for two of the dogs are presented in table 3. In the case of dog 39-191, a much greater effect was obtained when the vitamin A and alcohol were given together than when either was given alone. This effect, however, was

TABLE 3

*The effect of ethyl alcohol on the absorption of vitamin A in dogs.*

| No. of dog | 39–191 | | | 39–11 | | |
|---|---|---|---|---|---|---|
| Body weight (kg.) | 16 | | | 17 | | |
| Date | 5–22–40 | 5–27–40 | 6–3–40 | 5–27–40 | 5–22–40 | 6–3–40 |
| Treatment | 28,800 E.P.U. vit. A by mouth | 40 ml. 95% ethyl alcohol by stomach tube | 28,800 E.P.U. vit. A by mouth + 40 ml. 95% ethyl alcohol by stomach tube | 30,600 E.P.U. vit. A by mouth | 40 ml. 95% ethyl alcohol by stomach tube | 30,600 E.P.U. vit. A by mouth + 40 ml. 95% ethyl alcohol by stomach tube |
| *Serum: Vit. A per 100 ml. (E.P.U.[1])* | | | | | | |
| Before test | 276 | 255 | 230 | 142 | 224 | 188 |
| 4 hours after test | 569 | 447 | 671 | 438 | 428 | 410 |
| 9 hours after test | 717 | 460 | 748 | 630 | 478 | 515 |
| 12 hours after test | 856 | 608 | 1210 | 621 | 447 | 515 |
| 24 hours after test | 830 | 716 | 1493 | 459 | 272 | 468 |
| 30 hours after test | 793 | 584 | 1414 | ... | ... | 476 |

[1] Evelyn photoelectric unit.

not observed in the case of the other two dogs. It may be that, in some subjects, alcohol retards the absorption of the vitamin by decreasing the motility of the intestine, or that it causes rapid elimination of the vitamin by way of the intestinal tract. Some of our dogs were observed to defecate while alcohol was being injected by vein.

*The determination of the free and esterified forms of vitamin A of serum of dogs after administraton of alcohol*

The concentrations of free and of esterified vitamin A in the serum of three dogs which had received methyl alcohol were

determined. It was found that the rise in vitamin A was due almost wholly to the rise of the esterified form while the concentration of the free form remained practically unchanged. Table 4 gives the protocol of one experiment. We have not completed similar analyses of the serum of dogs treated with other alcohols.

Since the evidence available indicated that the mobilized vitamin A was derived from the stores in the body, the form in which the vitamin was present in these stores was investigated

TABLE 4

*The concentrations of free and esterified vitamin A in dog serum following the administration of methyl alcohol.*

| Dog 38–309. | 20 ml. methyl alcohol by vein. | | Wt. = 21 kg. |
|---|---|---|---|
| TIME | E.P U.[1] VITAMIN A PER 100 ML. OF SERUM | | |
| | Free | Esterified | Total |
| *hours* | | | |
| Before alcohol | 42 | 587 | 629 |
| 1½ after alcohol | 67 | 687 | 754 |
| 4 after alcohol | 23 | 1117 | 1140 |
| 19 after alcohol | 27 | 2462 | 2489 |
| 26 after alcohol | 21 | 2782 | 2803 |
| 44 after alcohol | 42 | 2300 | 2342 |
| 68 after alcohol | 50 | 1311 | 1361 |

[1] Evelyn photoelectric unit.

in two normal dogs. The dogs were sacrificed and samples of liver, kidneys, adrenals, retroperitoneal fat, and subcutaneous fat, taken for analyses. The results are summarized in table 5. It was found that in all of these tissues, with the exception of the kidneys of dog 39-11, the vitamin was present mainly as the ester.

The concentrations of the free and esterified forms of vitamin A in the urine of three dogs were also determined. As is evident from table 5, both forms are present and either may predominate.

TABLE 5

*Free and esterified vitamin A in the tissues and urine of dogs.*

| NO. OF DOG | | E P.U.[1] VITAMIN A PER 100 GM. OF SUBSTANCE | | |
| --- | --- | --- | --- | --- |
| | | Free | Esterified | Total |
| 39–11 | Liver | 123 | 1537 | 1660 |
| | Kidneys | 589 | 567 | 1156 |
| | Adrenals | 99 | 2725 | 2824 |
| | Subcutaneous fat | 149 | 285 | 434 |
| | Abdominal fat | 100 | 3884 | 3984 |
| 39–168 | Liver | 260 | 7524 | 7784 |
| | Kidneys | 311 | 1796 | 2107 |
| | Adrenals | 34 | 1541 | 1575 |
| | Subcutaneous fat | 45 | 1599 | 1644 |
| | Abdominal fat | 87 | 1143 | 1230 |
| | Urine | 20 | 6 | 26 |
| 40–80 | Urine | 77 | 2 | 79 |
| 40–104 | Urine | 4 | 13 | 17 |

[1] Evelyn photoelectric unit.

### DISCUSSION OF RESULTS

Our experiments clearly show that when dogs received methyl, ethyl, propyl or isopropyl alcohols either by mouth, vein, or injection into the peritoneal cavity, there was a prompt increase in the concentration of vitamin A in their serum. This increase was not due to increased absorption of the vitamin from the gastrointestinal tract, for the following reasons: (1) Dogs which had been fasting for 48 hours showed no decrease in the vitamin A mobilized; (2) the diets used in these experiments caused little variation in the vitamin A concentration of the serum during the course of the day, or even in the course of several days; (3) the gastrointestinal tracts of dogs receiving the diet contained little vitamin A; (4) in two dogs with fistulas of the thoracic duct, the vitamin A concentration of the lymph did not increase after the administration of the alcohol, but a rise in the vitamin A of the serum did occur.

We concluded that the mobilized vitamin A must have been derived from the liver and extrahepatic stores, for the follow-

ing reasons: (1) Almost all of the vitamin A present in these stores was in the form of the ester; (2) almost all of the mobilized vitamin A in the serum was in the form of the ester; (3) in two dogs which received daily injections of ethyl alcohol, there was a marked decrease in the vitamin A of their livers during the course of the experiment.

The increase of vitamin A in the serum cannot be ascribed to the conversion of carotene to the vitamin under the influence of alcohol, for carotene is present in the tissues of the dog in only minute amounts.

The presence of both free and esterified vitamin A in the tissues may lead to a better understanding of the metabolism of vitamin A by the body. Clausen et al. (40 c) showed that nearly all the vitamin A in the serum and urine of children was in the free form. Recently we determined the form of vitamin A in many tissues of man, cat, rabbit, guinea pig, chicken, gopher and monkey. A small amount of the free vitamin was always present, but when the concentration of the vitamin was relatively large, the greater part was in the ester form. It therefore appears that vitamin A is stored and transported mainly as the ester. The studies reported in this paper do not warrant conclusions being drawn regarding the function of the small amount of the free vitamin present in the tissues.

## CONCLUSIONS

After the administration of methyl, ethyl, propyl and isopropyl alcohols to dogs, there was a marked increase in the vitamin A concentration of the serum, the maximum rise usually being evident within 24 hours. The mode of administration of the alcohol, whether by mouth, vein, or injection into the peritoneal cavity, did not affect the results. It was shown that the effect was not due to increased absorption of the vitamin from the gastrointestinal tract. The vitamin A was mobilized from its storage places in the body, such as the liver and fat depots. Almost all of the mobilized vitamin A and the greater part of the vitamin present in the tissues of the dogs was found to be

in the form of the ester. There was, however, a small amount of free vitamin A present. It is therefore suggested that vitamin A must be in the free form in order to be utilized by the tissues, and that it is stored as the ester. In dogs which received daily doses of ethyl alcohol, the rise in the vitamin A concentration of the serum occurred each day. During the course of this experiment, the concentration of vitamin A in the liver decreased in the cases of two dogs whose original stores were low, but increased in the case of a third dog with initially higher stores.

## LITERATURE CITED

CLAUSEN, S. W., W. S. BAUM, A. B. McCOORD, J. O. RYDEEN AND B. B. BREESE 1940 a Mobilization of vitamin from its stores in the tissues by ethyl alcohol. Science, vol. 91, p. 318.

CLAUSEN, S. W., W. S. BAUM, J. O. RYDEEN, B. B. BREESE AND A. B. McCOORD 1940 b The determination of free and esterified vitamin A in mixtures. Am. Chem. Soc., Abstracts, 99th meeting.

CLAUSEN, S. W., A. B. McCOORD, W. S. BAUM, L. G. STEADMAN, J. O. RYDEEN AND B. B. BREESE 1940 c The determination of free and esterified vitamin A in human blood and urine. Am. Chem. Soc., Abstracts, 100th meeting.

CLAUSEN, S. W., B. B. BREESE, W. S. BAUM, A. B. McCOORD AND J. O. RYDEEN 1941 The effect of alcohol on the vitamin A content of blood in human subjects. Science, vol. 93, p. 21.

CLAUSEN, S. W., AND A. B. McCOORD 1938 The carotinoids and vitamin A of the blood. J. Pediat., vol. 13, pp. 635–650.

COLSON, Z. W. 1940 The effect of alcohol on vision. J. Am. Med. Assoc., vol. 115, p. 1525.

DANN, W. J., AND K. A. EVELYN 1938 The determination of vitamin A with the photoelectric colorimeter. Biochem. J., vol. 32, p. 1009.

McCOORD, A. B. 1939 Distribution of vitamin A in the body tissues of experimental animals. J. Pediat., vol. 14, p. 111.

McCOORD, A. B., AND E. M. LUCE-CLAUSEN 1934 The storage of vitamin A in the liver of the rat. J. Nutrition, vol. 7, p. 559.

PETT, L. B.  1940  Mobilization of vitamin A by alcohol. Science, vol. 92, p. 63.

SOLYANIKOVA, V. L.  1938  The influence of vitamin D on the vitamin A content of the blood and tissues of animals.  Biokhimiya 3, pp. 164–168. (Chem. Abs., vol. 34, p. 6335, 1940.)

YOUNG, G., AND G. WALD  1940  The mobilization of vitamin A by the sympathico-adrenal system.  Am. J. Physiol., vol. 131, p. 214.

# CYSTIC PITUITARY IN YOUNG CATTLE WITH VITAMIN A DEFICIENCY [1]

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TWO PLATES (FIVE FIGURES)

In connection with both pathological studies on tissues from cattle on deficient diets and certain physiological and histological studies on the pituitary gland, routine examinations have been made on this gland from young beef and dairy cattle either suffering from vitamin A deficiency or having a previous history of vitamin A depletion. In the glands examined a large cyst containing clear serum-like fluid has been found in most cases [2]. The cyst usually occupies space in or between the posterior and anterior lobes, often causing considerable pressure atrophy of the surrounding glandular parenchyma. In some cases the cyst appears to have formed in the residual lumen but in others it appears to be within the posterior lobe only, causing distortion of the entire gland.

References to this condition in cattle are limited. Moore ('39) briefly noted cysts in the pituitary of vitamin A deficient calves and suggests their presence as "further evidence of disturbance in the region of the cranial cavity" during this

deficiency. Sutton, Krauss and Hansard ('40) have also reported "accumulation of fluid in the cleft between the anterior and posterior lobes of the pituitary" in young bulls fed a vitamin A deficient ration for about 1 year. These latter workers reported an increase in alpha cells in the anterior lobe of such a pituitary and on assay this portion of the gland showed increased gonadotropic hormone content. This was believed to indicate compensatory activity in the pituitary resulting from injury to the testes caused by the deficiency of vitamin A. This view is consistent with the earlier findings of Sutton and Brief ('39) in vitamin A deficient rats in which gonadotropic activity of the pituitary was found to be higher than normal and the cellular changes in the gland were interpreted to be similar to those found after castration. The mechanism responsible for increased gonadotropin in the anterior lobe of vitamin A deficient bulls is difficult to explain on a basis of testis injury simulating castration since Bates, Riddle and Lahr ('35) using the immature male pigeon as the test animal found steers to be less potent in this factor than bulls. One of us (S. R. H.) and Dr. J. T. Bradbury have also recently found the bull to be more potent in this factor as indicated by the rabbit ovulation test.

None of the previous reports has emphasized either the finding of pressure atrophy or evidence of other degenerative changes in the pituitary of cattle suffering from vitamin A deficiency; such phenomena are described in the present work.

### EXPERIMENTAL OBSERVATIONS

During studies on the gonadotropic and lactogenic hormones of the anterior lobe of the pituitary more than 10,000 pituitaries have been collected from apparently normal cattle slaughtered at the Beltsville Research Center and at a meat packing plant in Baltimore, Maryland. In this group only four glands, and possibly a fifth, have been encountered that were definitely cystic. The rare occurrence of cystic pituitary glands in normal cattle is in marked contrast to the large number

found in a group of young cattle that developed varying degrees of vitamin A deficiency while on experimental rations.

In a series of fifteen vitamin A deficient animals of beef and dairy ancestry varying in age from the day of birth to 787 days of age, thirteen were found to have cystic pituitary glands. The beef animals consisted of one Shorthorn (cow 234), and a group of Shorthorn-Hereford crossbreds produced in connection with a study of the vitamin A requirement for reproduction. The dairy animals had been used in a study on the vitamin A requirement for growth and health of young animals. In this group one was a Jersey (steer 310-B) and the others were Holsteins. These cases are summarized in table 1.

The cystic pituitary of cow 234 was noted in a previous publication by Davis and Madsen ('41). This cow was depleted of vitamin A for a period of 125 days beginning at 23 days of age. Marked symptoms of deficiency appeared, including papilledema, low blood carotene, and low blood vitamin A, convulsions, diarrhea, night blindness and slow growth. The animal was then given enough high-grade alfalfa leaf meal of known carotene content to supply 120 µg. of carotene per kilogram of body weight per day. Within a short time the deficiency symptoms disappeared and apparently normal health was regained; however, the animal failed to grow normally. She conceived at 18 months of age and aborted during the eighth month of pregnancy. She was slaughtered 4 days after aborting and at this time the chief autopsy findings were small under-developed ovaries and a cystic pituitary gland. No specific cause for abortion was established. The pituitary of this cow (figs. 1, 2, and 3) is of special interest since the effects of the cystic condition were still apparent after a long period of adequate carotene intake. A considerable proportion of the functional anterior pituitary was replaced by the cyst and a large part of the remaining glandular parenchyma was replaced by fibrous connective tissue in which were scattered small groups of anterior lobe cells and smaller cysts. Many

*Occurrence of pituitary cysts in young beef and dairy cattle receiving an insufficient amount of vitamin A or carotene.*

| ANIMAL NO. | SOURCE AND AMOUNT OF VITAMIN A AND CAROTENE FED | | | AGE AT SLAUGHTER | DEFICIENCY SYMPTOMS | PITUITARY CYST |
|---|---|---|---|---|---|---|
| | Whole milk | Yellow carrots [1] | Carotene in oil [2] | | | |
| *Beef cattle* | *days* | *grams daily* | *µg./kg. body wt.* | *days* | | |
| Cow 234 | 23 | | | 787 | In calfhood only | Large |
| Heifer 166-1 | 103 | | | 103 | Papilledema after 30 days | Very large |
| Heifer 78-1 | 44 | | | 44 | Born blind; convulsions | Very large |
| Heifer 173-1 | 87 | | | 87 | Born blind; convulsions | Very large |
| Bull 173-2 | 31 | | | 31 | Born blind; convulsions | Very large |
| Heifer 29-1 | 0 | | | 0 | Died shortly after birth | Small |
| Bull 33-1 | 0 | | | 0 | Died shortly after birth | None |
| *Dairy cattle* | | | | | | |
| Steer P-2 | 98 [3] | | | 282 | Blind about 10 days | Very large |
| Steer 310-B | 30 | 60 after 30 days | | 180 | Blind about 17 days | Very large [4] |
| Steer P-16 | 4 | 60 to 7 months | | 540 | Blind right eye at 254 days; both at slaughter | Very large |
| Steer P-21 | 4 | 60 to 7 months | 29 after 7 months | 540 | Papilledema at 210 days | Large |
| Steer P-13 | 4 | 240 to 7 months | 29 after 7 months | 540 | Night blind; occasional convulsions | Medium |
| Steer P-22 | 4 | 240 to 7 months | | 540 | Blind several days | Very large |
| Steer 121-B | 4 | | 40 | 540 | Blind left eye at 180 days; both at slaughter | Medium |
| Steer 144-B | 4 | 1500 after 1 year | 145 1st month 116 2nd month 58 3rd month 29 4th to 12th mo. | 480 | Suddenly blind at 313 days | None |

[1] The carrots were grated and fed in milk for the first 30 to 60 days.
[2] The small amount of carotene furnished by timothy hay was deducted after the animals were 7 months of age and the balance was fed in oil solution.
[3] Whole milk was fed to 71 days and from 72 to 98 days equal parts of skimmed milk and whole milk were fed, after which only skimmed milk was given.
[4] Cyst contained 0.75 ml. of serum-like fluid.

of the remaining glandular cells had pycnotic nuclei and appeared non-functional.

Heifer 166-1 was apparently normal at birth but developed symptoms of vitamin A deficiency at about 30 days of age and died 73 days later. Carotene in oil solution failed to help this animal when the deficiency symptoms were well established. The pituitary of this calf (fig. 4) had a very large posterior lobe cyst which nearly replaced this lobe and caused considerable compression of the anterior lobe. The mother of this calf received 60 µg. of carotene in alfalfa leaf meal per kilogram of body weight daily during gestation and lactation. Several other calves born to cows on the same level of carotene intake have remained apparently normal.

The other beef calves were from cows receiving 30 to 45 µg. of carotene per kilogram of body weight. Symptoms of vitamin A deficiency were evident in these calves at birth and all of them had cystic pituitaries except bull 33-1 which died within a few minutes after birth.

The dairy animals examined in this study were all born to cows receiving sufficient carotene for normal reproduction. The calves were castrated at 60 days of age. All of them except 310-B and P-2 were given skimmed milk from the fourth day to either 6 or 7 months of age, together with a grain mixture having the following percentage composition: whole white corn meal, 30; wheat bran, 20; soybean meal, 25; and linseed meal, 25. Steers 310-B and P-2 were given considerably more whole milk and their grain mixture contained yellow corn meal in place of white corn meal. All of them were given liberal amounts of late cut low-carotene U. S. no. 3 timothy hay.

All of the dairy animals developed symptoms of vitamin A deficiency (table 1). Steers 310-B and P-16 received the least carotene and had very large pituitary cysts. Steers P-13 and P-22 received apparently adequate amounts of carotene up to 7 months of age, but steer P-22 still developed a cystic pituitary when the carotene supplement was discontinued, and steer P-13 when an inadequate amount of carotene was given.

Forty micrograms of carotene in oil per kilogram of body weight daily failed to protect steer 121-B from vitamin A deficiency. Blindness was noted in the left eye when the animal was about 6 months of age, and it was blind in both eyes at time of slaughter. At autopsy the left optic nerve was found to be completely constricted in the region of the optic foramen, and was smaller than normal throughout its entire length. The right optic nerve was only slightly constricted. The cyst in the pituitary of this animal was not large and histologically, no evidence of injury to the glandular parenchyma was seen.

Steer 144-B finally became blind from vitamin A deficiency, but it had an apparently normal pituitary at autopsy. This animal received large amounts of carotene early in life. After 12 months of age 1.5 kg. of carrots were added to the diet but no improvement in vision resulted.

## DISCUSSION

The cases presented are consistent since they had either vitamin A deficiency at death or a previous history of vitamin A depletion, and all had cystic pituitary glands except steer 144-B and calf 33-1. Steer 144-B received more carotene than any of the other animals and from past experience one would have expected calf 33-1 to develop a cystic pituitary gland if it had survived. All of the animals, except the two calves which died shortly after birth, were known to have had visual defects and other symptoms confirming vitamin A deficiency. The high incidence of cystic pituitary glands in young cattle in which this deficiency has been experimentally produced suggests that this is a part of the pathology of vitamin A deficiency.

In the case of cow 234, the cyst persisted with little or no evidence of regeneration of functional anterior lobe in spite of a later adequate carotene intake. Steer 121-B shows that when the cyst of the pituitary is not large, histological evidence of injury to the surrounding glandular portions is lacking. When the cyst is large, however, enough of the glandular parenchyma is replaced or constricted to suggest partial

hypophysectomy. Results with steers P-13 and P-22 indicate that a cystic pituitary may develop in young cattle that are depleted after having received adequate amounts of carotene up to 7 months of age.

Moore ('39), and Moore and Sykes ('40) have shown that blindness in young bovines is associated with constriction of the optic nerve due to constriction of the optic foramen which in turn is probably due to increased intracranial pressure. Wolbach and Bessey ('40) have pointed out that a vitamin A deficiency in young animals (rats, guinea pigs, dogs), if established early, causes injury to the central nervous system. These authors suggest that this is due to growth of the nervous system not retarded by vitamin A deficiency, while the growth of bone, particularly endochondral growth of the bony enclosure of the central nervous system, is retarded. This results in mechanical injury to the brain, spinal cord, and nerve roots due to overcrowding.

It is highly probable that injury to the pituitary gland in calves results from the mechanism responsible for injury to both the optic nerve tracts and other parts of the central nervous system. Additional observations are needed to determine the relationship of age to the development of this condition, its effect on vital functions, and the effect of a period of deficiency in the calf on the subsequent usefulness of the animal.

## SUMMARY

Cases of cystic pituitary glands have been found in young beef and dairy cattle either suffering from vitamin A deficiency or with a history of early severe vitamin A depletion. The cysts occurred either in the residual lumen or within the posterior lobe, often causing compression of the gland and injury to the glandular parenchyma. No evidence of repair in a cystic pituitary was found in an animal that was vitamin A deficient early in life but later fed adequate amounts of carotene, suggesting that the injury of the gland may be permanent.

## LITERATURE CITED

BATES, ROBERT W., OSCAR RIDDLE AND ERNEST L. LAHR 1935 An assay of three hormones present in anterior pituitaries of seven types of cattle classified for age, sex and stage of reproduction. Am. J. Physiol., vol. 113, pp. 259–264.

DAVIS, RUSSELL E., AND LOUIS L. MADSEN 1941 Carotene and vitamin A in cattle blood plasma with observations on reproductive performance at restricted levels of carotene intake. J. Nutrition, vol. 21, pp. 135–146.

MOORE, L. A. 1939 Relationship between carotene, blindness due to constriction of the optic nerve, papillary edema and nyctalopia in calves. J. Nutrition, vol. 17, pp. 443–459.

MOORE, L. A., AND J. F. SYKES 1940 Cerebrospinal fluid pressure and vitamin A deficiency. Am. J. Physiol., vol. 130, pp. 684–689.

SUTTON, T. S., AND B. J. BRIEF 1939 Anatomical and physiological changes in the pituitary glands of vitamin A deficient rats. Ohio Agr. Expt. Sta. Bull. 610, pp. 1–26.

SUTTON, T. S., W. E. KRAUSS AND S. L. HANSARD 1940 The effects of vitamin A deficiency on the young male bovine. J. Dairy Sci., vol. 23, p. 574. (Abstract.)

WOLBACH, S. B., AND OTTO BESSEY 1940 Relative overgrowth of the central nervous system in vitamin A deficiency in young animals. Science, vol. 92, pp. 483–484.

## PLATE 1

### EXPLANATION OF FIGURES

1 Section of anterior lobe of the pituitary from cow 234. × 5.8. Note position of cyst and large (light stained) area of pressure atrophy.

2 Near edge of pituitary from region A of figure 1. × 152. Note connective tissue replacement and atrophy of glandular cells.

3 Region B of figure 1. × 284. Note dense connective tissue stroma and nests of glandular cells, some of which are degenerating.
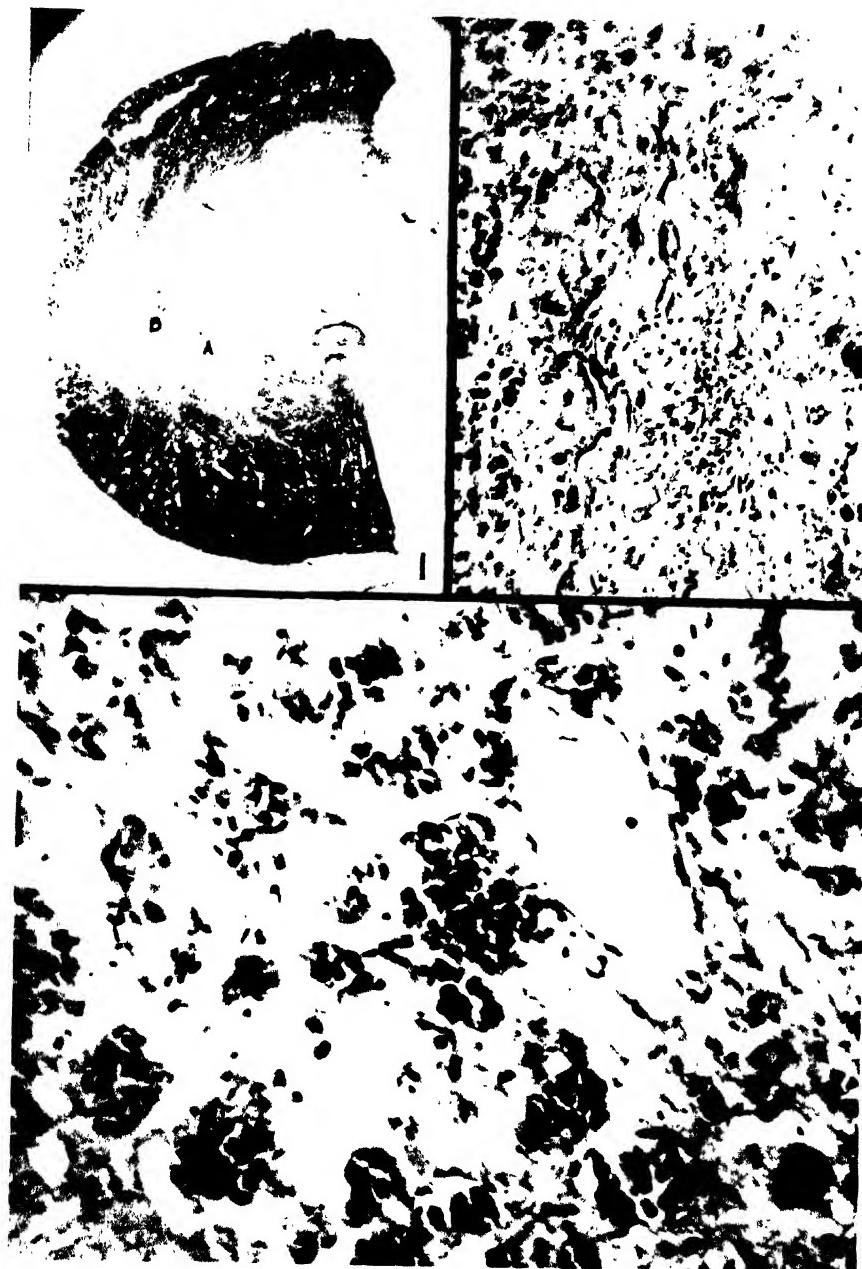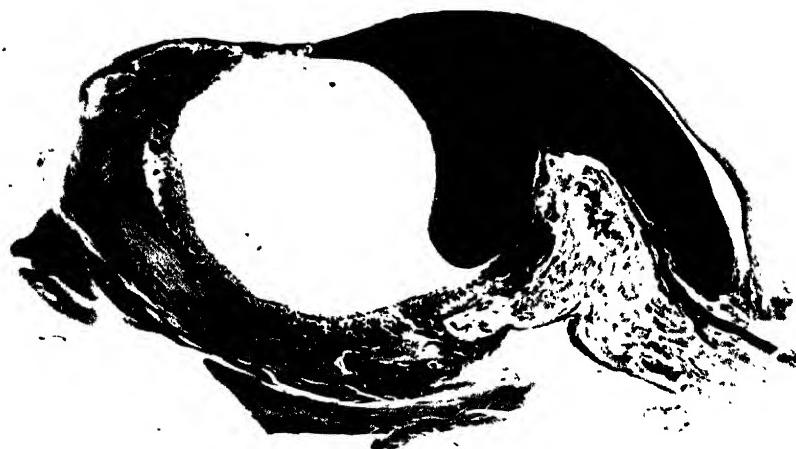
**4**



4 and 5   Sagittal section of pituitary from calves 166 1 and 173 1, respectively.
Note location of large cyst in posterior lobe with compression of anterior lobe.

# A STUDY OF THE ASCORBIC ACID REQUIREMENTS OF CHILDREN OF EARLY SCHOOL AGE [1]

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THREE FIGURES

The amount of ascorbic acid required for normal nutrition is not, as yet, well established. More detailed studies are needed at all age levels. Standards for the infant have been estimated, for the most part, either by determining the amount of vitamin C it takes to prevent the development of infantile scurvy, or by comparing the blood levels of breast-fed and artificially-fed infants with the relative amounts of ascorbic acid provided by the milk. While well-controlled, quantitative studies have been made on preschool children and adults, investigations on the school-age child have been principally of the survey type. In such studies the vitamin C content of the diet is only estimated, usually by dietary records or by inventory methods. This study was undertaken with the view of contributing quantitative data on the vitamin C requirements of children of early school age.

## EXPERIMENTAL

*Selection of subjects.* The subjects of this study were five children, two girls and three boys, between the ages of 7 and 12 years, living in a home for crippled and convalescent children. This institution was chosen because living conditions were such that it was possible to control all phases of the study. Of the children selected three had been under treatment for malnutrition, one for clubfoot, and one for rheumatic heart.

In all cases treatment had been successful, and the children were judged by the physician to be in a normal state of health. They were being retained in the institution until home conditions were favorable for their dismissal. The daily routine and activity of these children were comparable to that in any home. The children attended school regularly and played outside at recess and after school.

The physical status of the subjects is summarized in table 1. It is noted that all were of approximately average weight, or above, and that all gained substantial amounts during the

TABLE 1

*The physical status of subjects.*

| SUBJECT | AGE | | SEX | CONDITION FROM WHICH RECOVERED | BODY WEIGHT | HEIGHT | PER CENT OVER- OR UNDER- WEIGHT [1] | GAIN IN WEIGHT DURING STUDY |
|---------|-----|-----|-----|-------------------------------|-------------|--------|-----------------------------------|----------------------------|
| | *yrs.* | *mos.* | | | *kg* | *cm.* | | *kg.* |
| M. G. | 12 | 6 | F. | Malnutrition | 33.7 | 142.5 | — 6 | 1.5 |
| P. T. | 11 | 10 | M. | Rheumatic heart | 43.6 | 147.5 | + 13 | 1.8 |
| D. S. | 10 | 3 | F. | Malnutrition | 31.0 | 138.0 | — 3 | 2.5 |
| R. M. | 9 | 6 | M. | Malnutrition | 39.1 | 141.8 | + 12 | 2.3 |
| L. M. | 7 | 9 | M. | Clubfoot | 27.3 | 126.2 | + 4 | 2.2 |

[1] As judged by the Baldwin-Wood tables.

period of the study. The one who had had rheumatic heart gained 1.8 kg. in the 3 months. Weekly medical checks showed his heart condition to be completely controlled.

*General plan of the study.* The original plan for the study was to follow the saturation method used by Belser, Hauck and Storvick ('39) and to include, in addition, the determination of blood ascorbic acid. Since the initial blood levels of the children to be used in the study were found to be low (0.45 to 0.59 mg. per 100 cc.) it seemed wise to follow the changes in the blood levels as the intake was gradually increased, rather than to saturate the subjects at the beginning. The vitamin C content of the institutional diet was determined, therefore, and this amount used as the starting point for the investigation. To a basal diet providing 15 mg. of vitamin C, supplements of crystalline ascorbic acid [2] were added daily.

The investigation included eight experimental periods of 7 days each. During these periods the supplements of ascorbic acid ranged from 40 to 130 mg. Starting with 40 mg. the daily supplement was increased 20 mg. each week until the blood levels remained practically constant with the increased intake. Thereafter the supplements were gradually decreased until the 50 mg. level was reached for four subjects, and 90 mg. for the fifth.

The amounts of reduced ascorbic acid in the urine and blood plasma were determined on each level of intake. At the end of each period a test dose of 300 mg. vitamin C was given. The amount excreted in the urine during the 24-hour period following this test dose was used as one criterion for judging the state of saturation of each subject. Blood levels, urinary "resting level" and "retention values" are other criteria employed in the final analyses.

*Dietary control.* Chemical analysis of the institutional diet showed that the vitamin C was provided, for the most part, by a small glass of fruit juice, one serving of fresh fruit, and a quart of raw milk. The rest of the food furnished little ascorbic acid, due probably to the methods of preparation employed. Lettuce and cabbage, for example, were shredded 5 or 6 hours before serving, and most vegetables were overcooked. Potatoes stood in water both before and after cooking. They were then well aerated in an electric mixer and kept on the steam table for a considerable time. As a result all the vitamin C was destroyed.

The diet, on the whole, met the usual standards for adequacy with the possible exception of thiamine and vitamin C. The low blood levels of ascorbic acid indicated a shortage of this vitamin, even though the diet provided an average of 50 mg. daily.

Few changes were necessary, then, in setting up the experimental diet. Fruit juices and raw fruits and vegetables were eliminated and replaced by canned fruits, juices, and vegetables of low vitamin C content. The basic diet which was planned to furnish 15 mg. ascorbic acid, was made up from the foods listed in table 2. The weighed foods were always given in the same amount, with the exception of the milk

which varied from 750 to 1000 gm., depending upon the vitamin C content of the rest of the diet.

*Collection of urine.* For quantitative work Todhunter and Fatzer ('40) and Richardson and Mayfield ('40) have pointed out the necessity of using 24-hour urinary excretions. During this investigation the daily urinary output was collected and analyzed for 3 or 4 days on each level of intake. In addition, analyses were made on the 24-hour collection following each test dose.

To preserve the ascorbic acid in the urine 5 N sulphuric acid and 8-hydroxyquinoline were used as suggested by Sendroy and Miller ('39). By using brown bottles for the urine, acidifying the samples immediately, and keeping them in the refrigerator, losses were kept within 2%.

TABLE 2

*Foods used in the basal diet with amount of ascorbic acid contributed by each food.*

| FOOD | AMOUNT | ASCORBIC ACID | FOOD | AMOUNT | ASCORBIC ACID |
|---|---|---|---|---|---|
| *Foods weighed* | *gm.* | *mg.* | | *gm.* | *mg.* |
| Fruit juices | | | Vegetables, canned | | |
| Peach | 120 | 0.7 | Carrots | 60 | 0.3 |
| Pear | 120 | 0.4 | Beets | 70 | 3.0 |
| Plum | 120 | 0.3 | Beans, green | 60 | 1.8 |
| Apple | 120 | 1.2 | Beans, Lima | 60 | 0.4 |
| Dried fruit (stewed) | | | Potatoes, mashed | 110 | .. |
| Apricots | 80 | 2.9 | Milk, raw | 750–1000 | 7.5–12.0 |
| Peaches | 80 | 2.3 | Cocoa | 200 | 0.5 |
| Pears | 80 | 0.3 | Cod liver oil | 1 tsp. | .. |
| Apples | 80 | 0.2 | | | |
| Raisins | 80 | 0.2 | *Foods served ad lib.* | | |
| Prunes | 80 | 0.3 | Meats | daily | |
| Canned fruit | | | Beef | | |
| Pear, Bartlett | 80 | 0.8 | Veal | | |
| Juice | 20 | 0.3 | Lamb | | |
| Cherries | | | Bacon | | |
| (Royal Ann) | 80 | 2.2 | Chicken | | |
| Juice | 30 | 1.8 | Eggs | { 4–6 times | |
| Plums (Lombard) | 60 | 0.6 | | per week | |
| Juice | 30 | 0.4 | Cereals | daily | |
| Grapes (white) | 60 | 1.2 | Bread | daily | |
| Juice | 30 | 0.8 | | | |
| Apple Sauce | 100 | 0.7 | | | |

*Chemical methods.* Because of the impossibility of taking the photoelectric colorimeter to the institution it was necessary to use the titration method for determining ascorbic acid. Preliminary tests showed, however, that by the addition of a buffer to the acidified urine the rapid fading of the end-point was prevented and the results checked within 2% of the values obtained with the photoelectric colorimeter. The 2,6-dichlorophenolindophenol used as an indicator was standardized daily by the procedure of Menaker and Guerrant ('38). For food and urine analyses the methods of Bessey and King ('33) were used. The micro-technique with the Farmer and Abt apparatus ('36) was employed for all blood determinations. Fasting blood plasma separated from capillary blood was used, special care being taken to prevent any hemolysis.

All solutions were prepared with water redistilled in glass. A fluorescent daylight lamp was used as a source of light for titrating.

### RESULTS AND DISCUSSION

As a basis for interpreting the results from these blood and excretion studies in terms of the requirements, three criteria have been used. These are based upon (1) the "retention" or "utilization" of ascorbic acid, (2) the response to a 300-mg. test dose, and (3) the concentration of ascorbic acid in the blood. Blood levels above 0.7 mg. per 100 cc., the level recommended as normal by Goldsmith and Ellinger ('39) and Wortis et al. ('38), have been used in this study to indicate a satisfactory state of nutrition in respect to vitamin C.

In addition to these three indices for estimating the ascorbic acid requirements, other methods are included in the discussion. These will provide a means of comparing the results with other reports found in the literature. Urinary "resting level," "saturation" as interpreted by Belser, Hauck and Storvick ('39) and blood levels higher than 0.7 mg. per 100 cc. have been included.

*"Retention values."* One method of interpreting the results from urinary excretion studies in terms of the requirements is based upon the "retention value," that is, the difference between the intake of ascorbic acid and that excreted in the urine. With preschool children, Hathaway and Meyer ('41)

demonstrated the constancy with which the body utilized ascorbic acid. They found the mean "retention" for four children to be 23 mg. on intakes ranging from 31 to 50 mg. This represented 74% of the intake at the 31 mg. level, the minimum intake on which maximum "retention" was obtained.

The tendency for the body to "retain" a nearly constant amount of ascorbic acid was observed with the subjects on this study. As shown in table 3 there is little variation in the

TABLE 3

*Average daily urinary excretion and average "retention" of ascorbic acid on various levels of intake.*

| PERIOD | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 6 | 7 | 8 | AVERAGE AND S D. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Intake—mg. | 55 | 75 | 95 | 115 | 135 | 105 | 85 | 65 | 145 | 125 | 105 | |
| "Excretion" mg. | | | | | | | | | | | | |
| M. G. | 19 | 32 | 51 | 70 | 80 | 62 | 40 | 37 | | | | |
| P. T. | 11 | 26 | 37 | 69 | 86 | 60 | 39 | 25 | | | | |
| D. S. | 19 | 32 | 55 | 80 | 95 | 65 | 47 | 34 | | | | |
| R. M. | 12 | 29 | 49 | 75 | 95 | 62 | 48 | 25 | | | | |
| L. M. | 21 | 44 | 54 | 67 | 100 | | | | 106 | 88 | 79 | |
| Average | 16 | 33 | 49 | 72 | 91 | 62 | 42 | 30 | | | | |
| "Retention"[1] mg. | | | | | | | | | | | | |
| M. G. | 36 | 43 | 44 | 45 | 55 | 43 | 45 | 28 | | | | 42 ± 7 |
| P. T. | 44 | 49 | 58 | 46 | 49 | 45 | 46 | 40 | | | | 47 ± 5 |
| D. S. | 36 | 43 | 40 | 35 | 40 | 40 | 38 | 31 | | | | 38 ± 3 |
| R. M. | 43 | 46 | 46 | 40 | 40 | 43 | 37 | 40 | | | | 42 ± 3 |
| L. M. | 34 | 31 | 41 | 38 | 35 | | | | 39 | 37 | 26 | 35 ± 5 |
| Average | 39 | 42 | 46 | 43 | 43 | 43 | 43 | 35 | | | | |

[1] Value does not include the two 24-hour specimen following the test dose

amounts "retained" by each individual on the different levels of intake tested. The values appear to be related to the weight of the children rather than to the age. They vary from 35 ± 5 mg. for L. M., the smallest subject, to 47 ± 5 mg. for P. T., the largest.

This relationship of intake to excretion and "retention" is further illustrated in figure 2. The daily urinary excretion of ascorbic acid varies in direct proportion to the intake. It is apparent, however, that the amount "retained" varies but
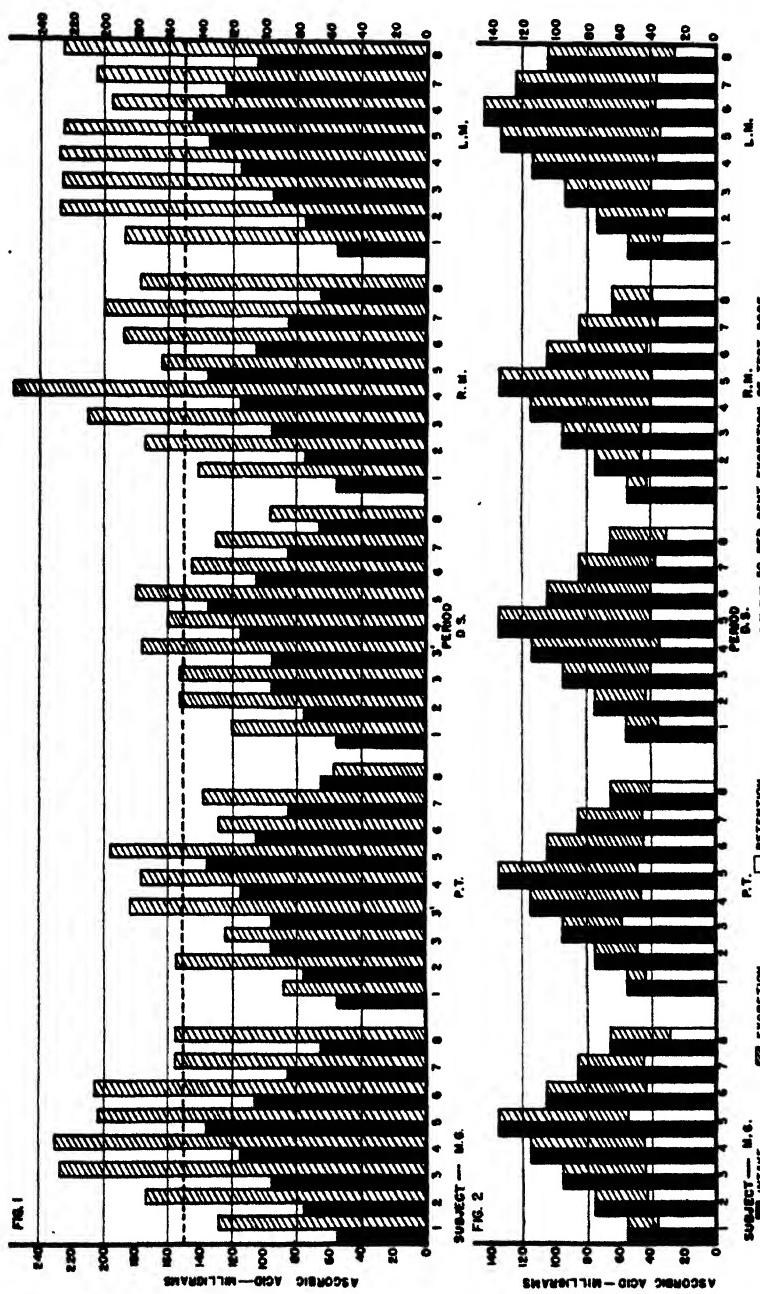
Fig. 1  24-hour excretion of ascorbic acid in response to a 300-mg. test dose following various levels of ascorbic acid intake. The black bars represent the previous intake, and the striped bars, the excretion after the test dose. 50% of the test dose is indicated by the broken line at the 150 mg. level.

Fig. 2  Average daily excretion and "retention" of ascorbic acid on various levels of ascorbic acid intake.  The black bars represent the daily intake; the striped bars, the excretion; and the white bars, the "retention."

little. The lowest intake on which each subject "retained" an amount of ascorbic acid equivalent to the average "retention value" ranges from 55 mg. for two of the children to 75 mg. for the other three. The "retention" at these levels of intake represents 57 to 77% of the ascorbic acid ingested.

*"Resting level."* The "resting level" or amount excreted daily in the urine has been used as another index for measuring the state of vitamin C nutrition. Van Eekelen and associates ('37) considered a "resting level" of 40 mg. indicative of tissue saturation. The average daily excretions for the five subjects on this study are reported in table 3. The ascorbic acid in the urine varied directly in proportion to the intake. The average excretion increased from 16 mg. on an intake of 55 mg. of vitamin C to 91 mg. when 135 mg. was ingested. An output of 40 mg. resulted from an intake of 75 mg. for one subject and 85 mg. for the other four.

*Response to a test dose.* A more widely accepted measure of vitamin C nutrition is the so-called "saturation test." By this method a large quantity of ascorbic acid is administered — orally, subcutaneously, or intravenously — and the amount excreted in the urine determined. A large percentage return of the test dose within 24 hours has been interpreted as indicating saturation. The exact percentage used by different workers, however, varies in respect to the size of the test dose given, the method of administration, and the length of the collection period. For this study a 300-mg. test dose of ascorbic acid was given orally, and a 50% return in the urine during the subsequent 24-hour period was used as the criterion for judging saturation.

The relationship between the intake and the response to the test dose is illustrated in figure 1. The youngest subject, L. M., returned more than 50% of the test dose on the lowest level of intake, that is, 55 mg. On all other levels he remained saturated. Sixty-five milligrams were required by M. G. and R. M. to produce the same response. They, too, remained saturated on intakes above 65 mg. The results for P. T. and D. S., however, were variable. In each case 75 mg. was the

lowest intake upon which 50% of the test dose was excreted. It will be noted that P. T. excreted less of the test dose on the 95 mg. level than on the 75 mg. intake. Upon repeating the test, the percentage return was greater. Similar variations have been observed in studies on adults (Belser, Hauck and Storvick, '39; Todhunter and Robbins, '40; and Fincke and Landquist, '42). The figures from the present study are in agreement with the findings of Koch ('41). With children of early school age she found that her subjects required 52 to 72 mg. of ascorbic acid before they excreted 50% of a 300 mg. test dose.

In figure 1 it is shown, also, that P. T. and D. S. always excreted less of the test dose than the others. The procedure followed by Belser, Hauck and Storvick ('39) rules out to some extent these differences due to individual variations. In general, they completely saturate the subject and then determine his individual response to the test dose. The value thus obtained is used to indicate his saturation response. It is questionable whether the data from the present study may be interpreted on the basis of this technique inasmuch as the subjects were not saturated at the beginning. We may assume, however, that they were all saturated at the three highest levels of intake, for the ascorbic acid level of the blood remained constant. With this interpretation, where each child sets his own standard for response to the test dose, three of the subjects required the same amount of vitamin C for saturation as was needed when the criterion consisted of 50% return of the test dose; that is, P. T., D. S., and R. M. required an intake of 75, 75, and 65 mg., respectively, to produce saturation. For the other two children the results were higher; 95 mg. was needed by M. G., and 75 mg. by L. M. The latter subject was not tested on an intake of 65 mg.

*Blood levels.* The amount of ascorbic acid found in fasting blood is believed by some to be the most accurate measure of the state of vitamin C nutrition. Here again there is a wide disagreement as to the level which represents tissue saturation. Faulkner and Taylor ('38) and Ralli et al. ('39) agree

that the renal threshold for ascorbic acid is about 1.3 to 1.4 mg. per 100 cc. It is at this blood level that the tissues are saturated; levels below this indicate varying degrees of unsaturation. On the other hand, there seems to be little agreement as to how low the blood levels may go without serious nutritional effects. Goldsmith and Ellinger ('39) and Wortis, Liebmann and Wortis ('38) believe 0.7 mg. per 100 cc. to be normal. The committee on vitamins of the American Academy of Pediatrics (Butler et al., '40) suggests that a
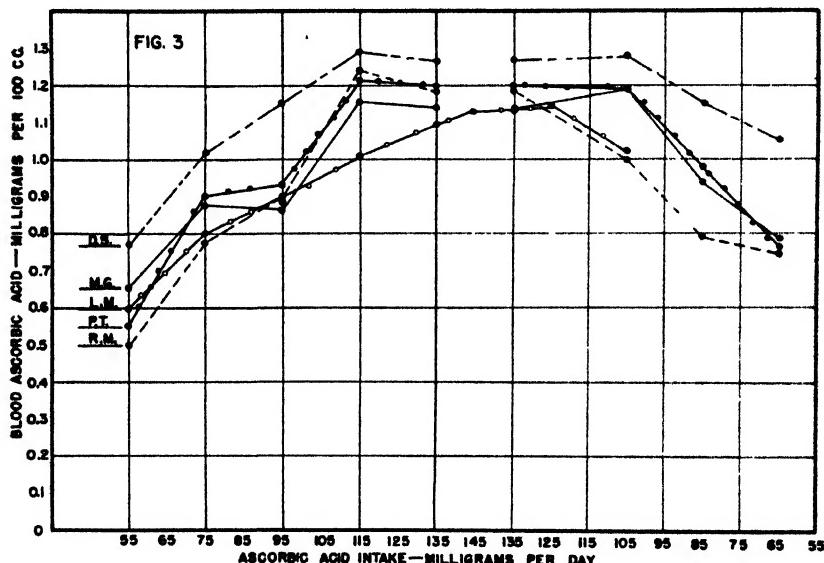


Fig. 3  Ascorbic acid concentration of the blood plasma of five subjects on varying levels of ascorbic acid intake.

serum or plasma concentration of 0.6 mg. or more per 100 cc. indicates a satisfactory state of vitamin C nutrition. Kajdi et al. ('39) believe that a blood level below 0.6 mg. per 100 cc. shows a serious depletion of body reserves. Whether these variations are due to differences in technique, or whether they are definite individual variations is difficult to determine.

At the beginning of the present study the blood levels of the children were between 0.5 and 0.6 mg. per 100 cc. The response of the blood plasma to graded amounts of vitamin C is illustrated in figure 3. The plasma values increased in

direct proportion to the intake until a plateau was reached at levels of 1.15 to 1.29 mg. per 100 cc. The lowest intakes which produced the maximum blood levels — the threshold value — were 105 mg. for three of the subjects, 115 mg. for the fourth, and 125 mg. for the fifth. As the intake was decreased below these amounts the plasma ascorbic acid likewise dropped.

To maintain blood values of approximately 0.9 mg. per 100 cc. the intake ranged from 65 to 95 mg. The two who required the highest intake to maintain this level, R. M. and L. M., were the ones who excreted the largest amount in response to the test dose. Also, their blood ascorbic acid rose more slowly than did that of the others.

On the lowest level of intake, 55 mg., the blood levels varied from 0.49 to 0.76 mg. per 100 cc. These individual variations in blood values were noted on all levels of intake. With adult subjects, Storvick and Hauck ('42) report variations even greater than these. If a blood value below 0.6 mg. per 100 cc. indicates a depletion of body reserves, then it would appear that the two largest subjects, P.T. and R.M., were depleted on the 55 mg. allowance. Only one subject, D. S., reached the plasma level of 0.7 mg. per 100 cc. on this intake. At least 65 mg. were required by all the others to bring the blood to 0.7 mg. per 100 cc., the concentration used in this study to indicate a satisfactory state of nutrition in respect to ascorbic acid. These results are in close agreement with those of Shelby ('41). In a study of thirty children, 6 to 12 years of age, she found that from 52 to 72 mg. were needed to maintain blood levels above 0.7 mg. per 100 cc. On the other hand, Bessey and White ('42) report that about 80% of their children had blood levels above 0.7 mg. per 100 cc. on intakes of 45 mg. This amount of ascorbic acid was estimated, however, on the consumption of citrus fruit and tomatoes only. Their results could better be compared then with the supplement alone used in the present study, and on this basis the results agree very well.

Of special interest are the blood levels corresponding to the "saturation" level. When a 50% excretion of the test dose

in 24 hours was used as the criterion for judging saturation, the blood ascorbic acid of the five subjects ranged from 0.60 to 1.05 mg. per 100 cc. These values are all below the renal threshold. In fact, some are below the levels suggested by some workers as being necessary for good nutrition.

### COMPARISON OF REQUIREMENTS ON THE BASIS OF DIFFERENT METHODS OF ANALYSIS

The requirements as obtained by the several methods used in the interpretation of the data are summarized in table 4.

TABLE 4

*Ascorbic acid requirements of five children as based upon various criteria for judging adequacy.*

| CRITERIA USED FOR JUDGING ADEQUACY | SUBJECT | | | | | |
|---|---|---|---|---|---|---|
| | M. G. | P. T. | D S. | R. M. | L. M. | Average |
| | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| Intake to maintain average "retention" value [1] | 75 | 75 | 75 | 55 | 55 | 67 |
| Intake to maintain "resting level" of 40 mg. urinary excretion | 85 | 85 | 85 | 85 | 75 | 83 |
| Intake to insure "saturation" a. As indicated by 50% excretion of a 300-mg. test dose [1] | 65 | 75 | 75 | 65 | 55 | 69 |
| b. As indicated by method of Belser, Hauck, Storvick | 95 | 75 | 75 | 65 | 75 | 77 |
| Intake to maintain blood levels a. Of 0.7 mg. or above [1] | 65 | 65 | 55 | 65 | 65 | 63 |
| b. Of 0.9 mg. or above | 75 | 75 | 65 | 95 | 95 | 81 |
| c. Of maximum value | 105 | 105 | 105 | 115 | 125 | 109 |

[1] The requirements for our subjects have been based upon these three indices.

Individually and collectively there is a wide range in the results. Including all methods of interpretation used, 65 mg. was the least value obtained for the requirements of the two oldest children, M. G. and P. T. Their blood levels were maintained above 0.7 mg. per 100 cc. at this level, and M. G. excreted 50% of the test dose in 24 hours. On all other bases, however, their requirements were higher. With the three

youngest children, 55 mg. was the least required. This was the lowest level providing maximum "retention" for two of the subjects. One of these, L. M., also excreted 50% of the test dose on this amount. Subject D. S. was the only child, however, who maintained a blood level above 0.7 mg. per 100 cc. when receiving 55 mg.

On the basis of the different criteria used it appears that an intake of 65 mg. for the two youngest children, and 75 mg. for the three oldest, would be sufficient to (1) maintain a blood level above 0.7 mg. per 100 cc., (2) provide for the maintenance of the average "retention" value and (3) insure saturation on the basis of a 50% excretion of a 300-mg. test dose. This represents a range of 1.7 to 2.4 mg. per kilogram of body weight. With other means of interpretation the requirements would be higher. Seventy-five to 85 mg., for instance, would be necessary to allow for a "resting level" of 40 mg. Amounts up to 95 mg. are needed to maintain blood levels of 0.9 mg. per 100 cc. In order to reach the threshold values these children required from 105 to 125 mg. of ascorbic acid.

### SUMMARY

The vitamin C requirements of five children between the ages of 7 and 12 years were studied. Blood values and urinary excretion of ascorbic acid were determined on levels of intake ranging from 55 to 145 mg. Following each level of intake a 300-mg. test dose was given to determine the state of tissue saturation. The amount "retained" by the body, the blood concentration, and the response to the test dose were used as criteria for judging the nutrition of each subject in respect to vitamin C.

Sixty-five milligrams of ascorbic acid were required by the two youngest children, and 75 mg. by the three oldest, to promote saturation on the basis of a 50% excretion of the test dose in 24 hours. These amounts were sufficient, also, to maintain blood levels above 0.7 mg. per 100 cc., and to allow for average "retention." With other methods of interpretation the requirements varied from these amounts up to 125 mg.

ACKNOWLEDGMENT

## LITERATURE CITED

BELSER, W. B., H. M. HAUCK AND C. A. STORVICK 1939 A study of the ascorbic acid intake required to maintain tissue saturation in normal adults. J. Nutrition, vol. 17, p. 513.

BESSEY, O. A., AND C. G. KING 1933 The distribution of vitamin C in plant and animal tissues and its determination. J. Biol. Chem., vol. 103, p. 687.

BESSEY, O. A., AND R. L. WHITE 1942 The ascorbic acid requirements of children. J. Nutrition, vol. 23, p. 195.

BUTLER, A. M., S. W. CLAUSEN, F. F. TISDALL AND W. W. WADDELL 1940 Report of the committee on vitamins of the American Academy of Pediatrics. Evanston, Illinois.

FARMER, C. J., AND A. F. ABT 1936 Determination of reduced ascorbic acid in small amounts of blood. Proc. Soc. Exp. Biol. and Med., vol. 34, p. 146.

FAULKNER, J. M., AND F. H. L. TAYLOR 1938 Observations on the renal threshold for ascorbic acid in man. J. Clin. Invest., vol. 17, p. 69.

FINCKE, M. L., AND V. L. LANDQUIST 1942 Personal communication.

GOLDSMITH, G. A., AND G. F. ELLINGER 1939 Ascorbic acid in blood and urine after oral administration of a test dose of vitamin C. Arch. Int. Med., vol. 63, p. 531.

HATHAWAY, M. L., AND F. L. MEYER 1941 Studies on the vitamin C metabolism of four preschool children. J. Nutrition, vol. 21, p. 503.

KAJDI, L., J. LIGHT AND C. KAJDI 1939 A test for the determination of the vitamin C storage: Vitamin C index. J. Pediatrics, vol. 15, p. 197.

KOCH, P. 1941 Unpublished thesis, The University of Chicago.

MENAKER, M. H., AND N. B. GUERRANT 1938 Standardization of 2,6-dichlorophenolindophenol. Ind. and Eng. Chem., Anal. Ed., vol. 10, p. 25.

RALLI, E. P., G. J. FRIEDMAN AND S. SHERRY 1939 The vitamin C requirement of man estimated after prolonged studies of plasma concentration and daily excretion of vitamin C in three adults on controlled diets. J. Clin. Invest., vol. 18, p. 705.

RICHARDSON, J. E., AND H. L. MAYFIELD 1940 The amount of ascorbic acid excreted at each urination during twenty-four-hour periods. Am. J. Physiol., vol. 128, p. 583.

SENDROY, J. S., JR., AND B. F. MILLER 1939 Renal function as a factor in the urinary excretion of ascorbic acid. J. Clin. Invest., vol. 18, p. 135.

SHELBY, P. 1941 Unpublished thesis, The University of Chicago.

STORVICK, C. A., AND H. M. HAUCK 1942 Effect of controlled ascorbic acid ingestion upon urinary excretion and plasma concentration of ascorbic acid in normal adults. J. Nutrition, vol. 23, p. 111.

TODHUNTER, E. N., AND A. S. FATZER 1940 A comparison of the utilization by college women of equivalent amounts of ascorbic acid (vitamin C) in red raspberries and in crystalline form. J. Nutrition, vol. 19, p. 121.

TODHUNTER, E. N., AND R. C. ROBBINS 1940 Observations on the amount of ascorbic acid required to maintain tissue saturation in normal adults. J. Nutrition, vol. 19, p. 263.

VAN EEKELEN, M., A. EMMERIE AND L. K. WOLFF 1937 Ueber die Diagnostik der Hypovitaminosen A und C durch die Bestimmung dieser Vitamine im Blut. Zeitschr. fur Vitaminforsch, vol. 6, p. 150.

WORTIS, H., J. LIEBMANN AND E. WORTIS 1938 Vitamin C in the blood, spinal fluid and urine. J. A. M. A., vol. 110, p. 1896.

# CHEMICAL COMPOSITION OF TWENTY-TWO COMMON FOODS AND COMPARISON OF ANALYTICAL WITH CALCULATED VALUES OF DIETS *

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One of the greatest needs today when the science of nutrition is being put to a practical test is specific information upon the chemical composition of normal diets as they are eaten. In response to this need, the present paper has been designed to provide data which permit a comparison of the determined summation value of the individual fresh foods which make up the total daily diets with the analyzed mixed diets subsequent to culinary treatment and service (i.e., as eaten), and with the values calculated from standard food tables.

In a survey of the literature on the mineral content of common foods, Word and Wakeham ('38) have pointed out that, while the calcium, phosphorus and sulfur contents of some foods vary by only 25% or less, those of others may vary as, much as 200%, when the analyses of different authors are compared. This statement need not imply that the validity of the analyses is questionable, but rather that there are differences in composition, even among specimens of the same variety of foods. In fruits (McCance and Lawrence, '29; McCance, Widdowson and Shackleton, '36), vegetables (Bishop, '34; Coleman and Ruprecht, '35; Davidson and LeClerc, '36), and cereal grains (Greaves and Hirst, '29; Schumpf-Pierron, '32), these discrepancies have been at-

tributed to variety, type of soil and cultivation employed, amount of water available, and climate. Attempts to correlate variations in foods with several factors have been made but information is still fragmentary. Several investigators (Bassett and Van Alstine, '35; MacKay and Butler, '35; Hawks, Dye and Bray, '37; Gutman and Low, '39; Hummel, Shepherd and Macy, '40), describing the technique of conducting metabolic experiments, have emphasized the fact that because of the variability in the composition of foods, the diet given must be analyzed under the precise conditions of the experiment, if its mineral, or even its protein, contents are to be used with any assurance of accuracy.

This report presents the inconstancy in composition shown by chemical analyses of representative samples of twenty-two individual foods which were used in making up mixed dietaries for normal children: (1) a comparison of the determined chemical constituency with values calculated from standard tables of food values (Sherman, '41); and (2) a comparison of the records of intakes of carbon, nitrogen, fat, energy, cellulose, hemicellulose, lignin, calcium, magnesium, sodium, potassium, phosphorus, chlorine and sulfur, as calculated by summation of the values from chemical analyses of the individual foods, and as determined directly on composite food mixtures representing the total daily dietary.

<center>EXPERIMENTAL</center>

*Sources of foods.* Canned and packaged goods of the same brand (cornflakes, graham crackers, tomato juice, peanut butter and orange juice) were purchased in bulk. Perishable foods, such as lettuce, carrots, cabbage, and lean beef (ground shoulder), were purchased daily at a local market. Fresh white and whole wheat breads were purchased daily from one bakery. Wealthy (Michigan) apples and Michigan potatoes were used exclusively and procured from a single source. The milk supply was controlled by a creamery company [1].

[1] Mr. C. G. Reichle, plant manager of Borden Farm Products of Michigan, skillfully controlled the handling of all the milk used in the analyses.

*Preparation of individual foods for analysis.* Samples of each of the individual foods were dried in a manner designed to cause the least possible alteration of their constituents. Solid foods were dried in vacuo at 40° C.; liquid foods were dried from the frozen state in the cryochem apparatus (Flosdorf and Mudd, '38) under vacuum at pressures of 150–200 microns. After the solid foods had reached constant weight, the samples were pulverized in a ball mill and stored in a desiccator until analyzed.

*Preparation of food mixtures for analysis.* The preparation of the separate foods, including sampling and preparation for analyses, has been described elsewhere (Macy, '42; Teague, Galbraith, Hummel, Williams, and Macy, '42). To obtain an accurate sampling of the foodstuffs, within the range of variation in composition usually occurring in practice and representative of the average American dietary, foods obtained at different seasons during several years were analyzed. All but five of the foods used (sugar, salt, milk, potatoes, butter) were incorporated in mixed composite samples of the diets, although milk was included in one composite. The salt used was chemically pure NaCl and the sweet butter given was thoroughly washed with distilled water. Sugar, salt and butter were not analyzed but their values were added to those for the composite. In most instances milk and potato were analyzed separately and the values added to those for the food mixture.

Food mixtures which would represent actual daily diets were prepared (Bassett and Van Alstine, '35). Each day samples of every food in the diet were taken and placed in separate, covered glass jars; each sample approximated two-fifths of the total daily intake of that food. At the end of 5 successive days each jar contained both a mixture of one food equal to approximately twice the daily intake in quantity, and a sample from each day's intake. From the food jars two identical composites were prepared, each representing a complete dietary for 1 day. One of the duplicate composite samples was transferred to an evaporating dish, and dried to

constant weight at 60° C. in the presence of alcohol. The dry material was ground in a ball mill, which insured thorough mixing, and used for determinations of the calcium, magnesium, potassium, sodium, phosphorus and chlorine contents. The second sample of the complete dietary was ground in a food chopper, and one aliquot of the homogeneous mixture removed for the determination of nitrogen; this aliquot was digested with sulfuric acid and made to volume. Another aliquot, about one-tenth of the total, was dried to constant weight in vacuo at 40° C., after which the material was ground in the ball mill and used for the determination of sulfur, energy, fat, carbon, lignin, cellulose and hemicellulose.

*Methods of analyses.* The methods employed in the analyses were used as published by Macy ('42); nitrogen was determined by the boric acid modification of the Kjeldahl method (Scales and Harrison, '20); calcium was precipitated as the oxalate from acetic acid solution (Scott, '39); magnesium was precipitated from the filtrate remaining from the calcium determination, under the conditions suggested by Willard and Furman ('35); sodium was precipitated as sodium uranium zinc acetate, using Butler and Tuthill's ('31) modification of the Barber and Kolthoff method; the Kramer and Tisdall ('21) method for the determination of potassium was followed as described by Peters and Van Slyke ('32); phosphorus was determined gravimetrically by MacKay and Butler's modification of Mathison's method (Peters and Van Slyke, '32); chlorine was determined gravimetrically as silver chloride (Garelli, '32); and, sulfur was precipitated as barium sulfate, after the food samples had been oxidized in the Parr Adiabatic Oxygen Bomb [2] and heat of combustion determined. Fat was extracted from the vacuum dried material in a Soxhlet apparatus. Carbon was determined by the macro wet-combustion process (Scott, '39). Lignin, cellulose and hemicellulose were determined by the method of Williams and Olmsted ('35).

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[2] Parr Oxygen Bomb Calorimeters and Oxygen Bomb Sulfur Apparatus, Manual No. 117, Parr Instrument Co., Moline, Illinois.

Iron was determined by the o-phenanthroline method devised by Hummel and Willard ('38).

### RESULTS AND DISCUSSION

The mean values from the determinations for the energy, fat, carbon, nitrogen, calcium, magnesium, potassuim, phosphorus, sodium, chlorine, and sulfur contents per 100 gm. of edible portion of each of the twenty-two foods used in the diets are summarized in table 1. The widest variations from the standard tables were found in the determinations of sodium and chlorine. Since NaCl is a component of extra-cellular fluid in plant and animal tissues, the sodium and chlorine contents might be expected to be influenced to a greater extent by the composition of the soil and water in which the plant materials were grown, and by the degree of dehydration. Potassium, which is an intra-cellular constituent, differed less from previously reported values than any other mineral constituent determined. Greater consistency among intra-cellular constituents is further supported by the phosphorus and sulfur [3] determinations, which also are in good agreement with values previously reported.

Since standard tables are usually compiled from the averages of large numbers of determinations from widely different sources, it is of interest to determine whether the mean values of the two foods for which the largest number of samples were analyzed (banana, 14; milk, 25) approach more closely the values reported in the standard tables. Analyses of more samples would be expected to include a wider range of variability and yield mean results in closer agreement with the standard tables; conversely, a single sample might be an extreme variation from the mean of many determinations and would then have little significance, except with reference to the experiment in which the food was used.

[3] Since the amounts of sulfur were exceedingly small, greater accuracy was attained by determining and applying to the data a correction factor for the solubility of barium sulfate in water (which was used as a wash solution).

TABLE 1

Variation in analysis content of individual foods[1] (values in units per 100 gm. edible portion).

| | ENERGY | FAT | CARBON | NITROGEN | CALCIUM | MAGNESIUM | POTASSIUM | SODIUM | PHOSPHORUS | CHLORINE | SULFUR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cal. | gm. | gm. | gm. | mg. | mg. | mg. | mg. | mg. | mg. | mg. |
| Apples (not peeled) | 55 | 0.88 | 5.4 | 0.04 | 6 | 8 | 125 | 2 | 13 | 2 | 4 |
| Banana (peeled) | 86 | 0.24 | 8.7 | 0.13[2] | 5[2] | 33[2] | 387[2] | 5[2] | 28[2] | 110[2] | 8[2] |
| Beef | 184 | 8.06 | 22.0 | 3.14 | 10 | 13 | 290 | 70 | 148 | 63 | 162 |
| Bread (white) | 290 | 1.36 | 27.6 | 1.56 | 60 | 28 | 107 | 601 | 114 | 843 | 116 |
| Bread (whole wheat) | 283 | 1.51 | 28.4 | 1.68 | 64 | 67 | 203 | 601 | 217 | 857 | 126 |
| Cabbage | 24 | 0.12 | 2.4 | 0.21 | 44 | 12 | 160 | 24 | 16 | ... | 64 |
| Carrot | 43 | 0.16 | 4.1 | 0.10 | 26 | 18 | 320 | 58 | 30 | 144 | 17 |
| Cheese (Velveta) | 350 | 19.90 | 30.0 | 3.05 | 565 | 15 | 255 | 1440 | 875 | 1122 | 178 |
| Cornflakes | 384 | 0.25 | 37.8 | 1.14 | 7 | 37 | 98 | 1120 | 42 | 1633 | 115 |
| Egg | 146 | 7.32 | 12.8 | 1.70 | 42 | 2 | 120 | 128 | 165 | 124 | 155 |
| Graham Cracker | 372 | 4.34 | 36.6[2] | 1.12 | 46 | 62 | 384 | 670 | 157 | 516 | 116 |
| Lettuce | 24 | ... | 2.0 | 0.14[2] | 16 | 12 | 172 | 16 | 20 | ... | 12 |
| Milk (evaporated) | ... | ... | ... | 0.97 | 253 | 25 | 294 | 138 | 209 | 224 | 68 |
| Milk (fluid) | 70[2] | 3.45[2] | 6.2[2] | 0.50[2] | 118[2] | 11[2] | 148[2] | 50[2] | 90[2] | 108[2] | 30[2] |
| Orange Juice (fresh) | 40 | ... | 5.2 | 0.15 | 11 | 11 | 187 | 6 | 15 | 8 | 6 |
| Orange Juice (canned) | ... | ... | 49.1[2] | ... | 12 | 12 | 192 | 4 | 24 | 6 | 6 |
| Peanut Butter | 632 | 45.20 | 10.0[2] | 4.64 | 28 | 178 | 694 | 384 | 341 | 616 | 225 |
| Peas | 96 | 0.44 | 7.7 | 1.10 | 22 | 32 | 296 | 98 | 114 | 187 | 41 |
| Potatoes (peeled) | 76[2] | 0.10 | ... | 0.35 | 8[2] | 22[2] | 394[2] | ... | 60[2] | 34[2] | 41[2] |
| Spinach (canned) | 20 | ... | 2.2 | 0.29 | 53 | 63 | 208 | 378 | 344 | 460 | ... |
| Tomato Juice (canned) | 20 | ... | 43.3 | 0.13 | 3 | 10 | 225 | 255 | 17 | 387 | 13 |
| Honey | 293 | ... | 69.0 | ... | ... | ... | ... | ... | ... | ... | 13 |
| Gelatin | 467 | ... | 28.7 | 16.20 | ... | ... | ... | ... | ... | ... | 467 |

[1] Unmarked figures are average of five, (or fewer) triplicate analyses of samples.

[2] Average of six to fifteen triplicate analyses of samples.

[3] Average of fifteen to twenty-seven triplicate analyses of samples.

The food most constant in composition, in our experience, both with respect to variation within a given series, and by comparison of our results with those of others, is fluid cow's milk. Analyses agree with Sherman's standards within 3% for energy, calcium, phosphorus, potassium, sodium, and chlorine; magnesium and sulfur, which are present in extremely small quantities in milk, agree within −8 and −12%, respectively; nitrogen and fat agree within −11 and −12%, respectively. The samples of milk analyzed represented pooled samples from one herd of cows and the results of the analyses emphasize the value of careful control of the source of a material to be analyzed for mineral composition; furthermore, a pooled sample might be expected to approach the previously determined mean figures more closely than a sample from a single animal. The constancy of milk might be predicted from the fact that it is a body secretion; however, it is very susceptible to contamination, a factor which has been frequently mentioned as a possible reason for variability in food analyses. The analytical value for milk composition indicates that either contamination of the samples was of minor importance in introducing errors in the analyses or the contamination factor is about the same for all milks analyzed, including those reported herein and by others.

Banana, which is protected by a skin, making the fruit practically free from contamination, varied widely in composition in spite of the fact that the fruit for the analyses reported in this paper was selected for uniform quality and ripeness by an expert trained in handling this type of fruit [4]. The standard deviations of the analyses of banana demonstrate a wide range of variability for several constituents, in spite of their protective covering, and the average values differ widely from Sherman's figures. The determined values agreed with those of Sherman within 6% for phosphorus, magnesium and potassium and 20% for fat; −12% for energy and

[4] Mr. B. E. Reiff, resident manager, Fruit Dispatch Company, Detroit, assumed the responsibility of selecting and delivering the bananas so that they would be at comparable stages of ripeness when analyzed.

chlorine; —33% for nitrogen and sulfur; —38% for calcium; and —88% for sodium.

The fat contents of the foodstuffs analyzed varied rather widely from the standard figures. The few foods, other than butter and milk, which contained fat in appreciable amounts were concentrated types of foodstuffs which are difficult to sample accurately. In addition, they were not uniform as purchased, though every possible precaution was observed to control variations which might arise at the source of supply. For example, shoulder beef was recommended as the most uniformly lean meat to be obtained and all purchases were made from one butcher, but samples were found to vary as much as 1 to 15% in fat content. The energy values of the beef confirmed the difference in composition.

Table 2 illustrates the variability of lignin, cellulose and hemicellulose analyses of individual foods. Although the shortcomings of the methods for determining the complex carbohydrates are recognized, the values recorded do have merit in that our knowledge of these constituents in the human dietary is so fragmentary. There are no analogous figures in the literature with which to compare our values.

Table 3 permits comparison of the sum of the values for individual foods in the diets with the determination of the same constituent on the mixed dietary composite as eaten; in addition, the quantity of each dietary constituent is shown as calculated from standard tables. The data for the mixed composite are means of eleven triplicate analyses. The standard deviations (S. D.) have been calculated and recorded. The sum of the analyses of individual foods is within a range of 1 S. D. from the mean of the analyses of the composite, for fat, nitrogen, magnesium, sodium, potassium, chlorine, and energy; for calcium, phosphorus, and sulfur the sum of the individual analyses is more than 1 S. D. below the mean. The calcium intakes calculated from the standard tables are significantly higher than the determined values, while the sodium and chlorine intakes are decidedly lower.

TABLE 2

Complex carbohydrates of individual foods (milligrams per 100 gm. of edible portion).

| | NO. SAMPLES | LIGNIN | | | CELLULOSE | | | HEMICELLULOSE | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Min. | Max | Mean | Min. | Max. | Mean | Min. | Max. |
| Apple [1] (not peeled) (Wealthy) | 2 | 170 | 136 | 203 | 514 | 460 | 568 | 200 | 197 | 203 |
| Banana (peeled) | 3 | 416 | 344 | 462 | 153 | 60 | 234 | 125 | 105 | 154 |
| Bread (white) | 3 | 71 | 68 | 76 | 92 | 0 | 275 | 494 | 444 | 534 |
| Bread (whole wheat) | 3 | 451 | 290 | 592 | 627 | 470 | 740 | 1362 | 1060 | 1723 |
| Cabbage [1] | 3 | 87 | 60 | 104 | 531 | 504 | 552 | 385 | 360 | 428 |
| Carrot [1] (scraped) | 3 | 97 | 64 | 114 | 676 | 608 | 784 | 517 | 368 | 682 |
| Cornflakes | 3 | 184 | 117 | 223 | 599 | 453 | 757 | 366 | 327 | 430 |
| Graham Cracker | 3 | 299 | 178 | 433 | 460 | 264 | 567 | 741 | 383 | 1056 |
| Lettuce | 3 | 83 | 80 | 84 | 367 | 340 | 412 | 242 | 215 | 260 |
| Peanut Butter | 3 | 95 | 36 | 212 | 323 | 100 | 600 | 762 | 531 | 931 |
| Peas | 3 | 65 | 48 | 84 | 2049 | 1856 | 2340 | 707 | 548 | 944 |
| Potatoes [1] (peeled) | 9 | 31 | 7 | 72 | 246 | 0 | 417 | 110 | 33 | 273 |
| Spinach (canned) | 1 | 128 | 128 | 128 | 286 | 286 | 286 | 142 | 142 | 142 |
| Tomato Juice (canned) | 2 | 8 | 7 | 10 | 126 | 110 | 143 | 34 | 27 | 42 |
| Shredded Wheat | 1 | 797 | 797 | 797 | 1650 | 1650 | 1650 | 3473 | 3473 | 3473 |

[1] Michigan.

## TABLE 3

*Chemical composition of diet as determined by analysis of composites, analysis of the individual foods, and by calculation from standard tables.*

| | VALUES FROM ANALYSIS OF FOOD COMPOSITE | | PRECISION[1] OF CHEMICAL METHODS | SUM OF VALUES FOR INDIVIDUAL FOODS | | PER CENT DIFFERENCE COMPARISON | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | Mean | SD | % | Determined | Calculated[2] | I[3] | II[4] |
| Energy (Cal.) | 1429[5] | 78 | 1.3 | 1448[5] | 1398[6] | +4 | +1 |
| Fat (gm.) | 35.52 | 2.72 | 1.6 | 34.91 | 40.29 | −13 | −2 |
| Protein (N × 6.25) (gm.) | 64.5 | 2.8 | .. | 62.8 | 65.6 | −4 | −3 |
| Nitrogen (gm.) | 10.32 | 0.45 | 0.3 | 10.04 | 10.49 | −4 | −3 |
| Calcium (gm.) | 0.347 | 0.014 | 3.6 | 0.312 | 0.434 | −28 | −11 |
| Magnesium (gm.) | 0.228 | 0.014 | 3.8 | 0.224 | 0.255 | −12 | −2 |
| Sodium (gm.) | 1.933 | 0.036 | 1.0 | 1.962 | 0.789 | +149 | +1 |
| Potassium (gm.) | 2.283 | 0.063 | 1.1 | 2.299 | 2.374 | −3 | +1 |
| Phosphorus (gm.) | 0.981 | 0.041 | 2.4 | 0.923 | 1.109 | −17 | −6 |
| Chlorine (gm.) | 2.582 | 0.088 | 1.1 | 2.522 | 1.081 | +133 | −2 |
| Sulfur (gm.) | 0.771 | 0.054 | 2.6 | 0.698 | 0.706 | −1 | −10 |
| Total positive minerals (meq.[7]) | 178.6 | ... | .. | 178.2 | 137.7 | +29 | −0.22 |
| Total negative minerals (meq.[7]) | 177.9 | ... | .. | 168.3 | 139.0 | +21 | −6 |
| Excess of positive minerals (meq.[7]) | 0.7 | ... | .. | 9.9 | −1.3 | ... | ... |

[1] Determined by Analysis of Variance (Snedecor, '34).
[2] From Sherman ('41).
[3] Sum of individual foods as analyzed vs. sum of individual foods as calculated.
[4] Sum of individual foods as analyzed vs. analysis of composite.
[5] Heat of combustion.
[6] Fuel value (Sherman, '41).
[7] Milliequivalents.

In general, a fairly large constituent, such as potassium in plant materials, or calcium in milk and cheese, can be determined with relatively greater accuracy than a very small one, such as calcium, magnesium, phosphorus and sulfur in most foodstuffs. Analysis of composite diets decreases the chances of error from weighing and handling and conserves the time of the laboratory staff, resulting in a great reduction of expense, although physical or chemical characteristics of some foods indicate the advisability of separate analysis and addition of the individual values to the data for the composite. The wide variations that occur in the composition of individual foods emphasize the advantages of analyzing composite diets.

In composition, the composite samples were subject to less than the usual number of variations, although changing season necessitated the purchase of foods of different variety, grown under different conditions of soil and climate. Possibilities that must be considered are (a) minor differences in weights from one period to another; (b) varying degrees of dehydration due to storing the foods, both in market and refrigeration; and (c) inherent personal errors by the persons through whose hands each sample passed before it was ready for analysis. The precision of the chemical methods has been calculated and is recorded in table 3. In many series of analyses the accuracy of a micromethod was approached.

*Comparison of composite diets.* Table 5 gives summaries from eleven constituents of the six mixed dietaries itemized in table 4. The number of samples analyzed, the range of the determinations, and the mean, with its standard deviation and the standard error of the mean, are given. In a recent paper, Hawks et al. ('40) have indicated that the errors inherent in analyses of composite diet preparations are from sources other than manipulation of the foods. The standard error of the mean in the determination of nitrogen (table 5) is of the same order of magnitude as the errors found by these investigators when foods weighed on the same day were analyzed. This is particularly significant when it is remembered that each mean given in table 5 presents the average

of successive determinations of 5-day composite diets which were collected over several weeks at a time. The determinations of calorie content of the diets likewise have practically the same range as those of Hawks and associates ('40), thus supporting their conclusion that any errors found in analyses of composite diets are from a source other than sampling and manipulation.

The analytical results presented for six dietary mixtures indicate that the accuracy of sampling and chemical methods

TABLE 4

*Distribution of foods in the mixed diet composites* [1]

| FOOD | AMOUNTS OF INDIVIDUAL FOODS IN COMPOSITE DIETS Grams | | | | | |
|------|------|------|------|------|------|------|
|      | A | B | C | D | E | F |
| Apple | 100 | 100 | 100 | 100 | 100 | 100 |
| Banana | 100 | ... | ... | ... | ... | 150 |
| Beef, lean | 60 | 100 | 100 | 100 | 100 | 100 |
| Bread, white | 60 | 20 | 10 | 70 | 60 | 50 |
| Bread, whole wheat | 30 | 30 | 30 | 30 | 30 | 50 |
| Cabbage | 50 [2] | 25 | 25 | 25 | 25 | 25 |
| Carrot | 50 [2] | 25 | 25 | 25 | 25 | 50 |
| Celery | 20 | .. | .. | .. | .. | .. |
| Cornflakes | .. | 30 [2] | 10 [2] | 30 [2] | 10 [2] | 30 |
| Shredded wheat | 30 | 30 [2] | · 10 [2] | 30 [2] | 10 [2] | .. |
| Cheese, American | 15 | 15 | 15 | 15 | 15 | 20 |
| Egg, whole | 50 | 50 | 50 | 50 | 50 | 100 |
| Graham cracker | .. | 36 | 36 | 36 | 36 | 36 |
| Gelatin | .. | .. | .. | .. | .. | 3 |
| Honey | .. | .. | .. | .. | .. | 15 |
| Lettuce | 20 | 20 | 20 | 20 | 20 | 25 |
| Milk | 400 | .. | .. | .. | .. | .. |
| Orange juice | 100 | .. | .. | .. | .. | 100 |
| Peas | .. | .. | .. | .. | .. | 25 |
| Peanut butter | .. | 16 | 16 | 16 | 16 | 16 |
| Potato | 70 | 40 | 40 | 70 | 70 | .. |
| Tomato juice | 60 | 60 | 60 | 60 | 60 | 60 |

[1] Sugar, salt and butter were not included in the composites. The salt used was chemically pure NaCl; fresh, sweet butter was thoroughly washed with distilled water. In some of the composites, banana, orange juice, milk and potato were omitted, but these foods were analyzed separately and the values added to those for the composite.

[2] Cabbage and carrot were interchangeable in composite A. Cornflakes and shredded wheat were interchangeable in composites B, C, D, and E.

TABLE 5

*Chemical composition of mixed composite [1] diets as eaten.*

| | ENERGY [2] | FAT | NITRO-GEN | CAL-CIUM | MAG-NESIUM | SODIUM | POTAS-SIUM | PHOS-PHORUS | CHLO-RINE | SULFUR | IRON |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cal. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | | mg. |
| **Composite A** | | | | | | | | | | | |
| Samples [3] | 10 | ... | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | ... |
| Minimum | 1097 | ... | 8.03 | 0.903 | .274 | 1.151 | 2.107 | 1.253 | 1.674 | .667 | ... |
| Maximum | 1405 | ... | 9.08 | 1.052 | .332 | 1.702 | 2.604 | 1.543 | 2.541 | .981 | ... |
| Mean | 1258 | ... | 8.44 | 0.989 | .310 | 1.368 | 2.324 | 1.348 | 2.060 | .756 | ... |
| SD | 90 | ... | 0.12 | 0.037 | .008 | 0.180 | 0.153 | 0.075 | 0.298 | .082 | ... |
| SE$_m$ | 28 | ... | 0.03 | 0.008 | .002 | 0.040 | 0.034 | 0.017 | 0.067 | 0.18 | ... |
| **Composite B** | | | | | | | | | | | |
| Samples [3] | 12 | 15 | 12 | 12 | 13 | 13 | 12 | 11 | 11 | 12 | 11 |
| Minimum | 857 | 23.4 | 7.02 | .223 | .152 | 0.788 | 1.124 | .581 | 1.174 | .435 | 6.38 |
| Maximum | 957 | 32.0 | 7.84 | .254 | .185 | 1.004 | 1.348 | .635 | 1.358 | .537 | 7.29 |
| Mean | 894 | 27.1 | 7.37 | .240 | .166 | 0.892 | 1.242 | .610 | 1.272 | .494 | 6.89 |
| SD | 22.5 | 2.6 | 0.28 | .016 | .010 | 0.061 | 0.078 | .019 | 0.057 | .029 | 0.28 |
| SE$_m$ | 6.5 | 0.7 | 0.08 | .005 | .003 | 0.017 | 0.022 | .006 | 0.017 | .008 | 0.08 |
| **Composite C** | | | | | | | | | | | |
| Samples [3] | 12 | 12 | 12 | 11 | 12 | 12 | 11 | 11 | 11 | 11 | 11 |
| Minimum | 754 | 25.4 | 6.78 | .209 | .128 | .694 | 1.133 | .505 | 0.975 | .428 | 5.92 |
| Maximum | 903 | 31.9 | 7.39 | .266 | .163 | .854 | 1.348 | .564 | 1.118 | .530 | 6.69 |
| Mean | 805 | 27.3 | 7.06 | .237 | .147 | .781 | 1.236 | .537 | 1.053 | .472 | 6.27 |
| SD | 30.2 | 1.9 | 0.34 | .017 | .012 | .051 | 0.080 | .012 | 0.042 | .039 | 0.31 |
| SE$_m$ | 8.7 | 0.6 | 0.10 | .005 | .003 | .015 | 0.024 | .004 | 0.013 | .012 | 0.09 |
| **Composite D** | | | | | | | | | | | |
| Samples [3] | 17 | 20 | 15 | 16 | 17 | 17 | 17 | 17 | 16 | 15 | 15 |
| Minimum | 1014 | 25.5 | 7.88 | .250 | .170 | 1.051 | 1.267 | .632 | 1.580 | .517 | 6.82 |
| Maximum | 1112 | 34.0 | 8.50 | .316 | .207 | 1.267 | 1.571 | .739 | 1.709 | .597 | 8.91 |
| Mean | 1059 | 29.3 | 8.24 | .284 | .186 | 1.192 | 1.430 | .689 | 1.653 | .572 | 7.69 |
| SD | 37.7 | 2.7 | 0.06 | .024 | .013 | 0.054 | .096 | .039 | 0.039 | .031 | 0.61 |
| SE$_m$ | 9.2 | 0.6 | 0.02 | .006 | .003 | 0.013 | .023 | .009 | 0.010 | .008 | 0.16 |
| **Composite E** | | | | | | | | | | | |
| Samples [3] | 5 | 5 | 6 | 6 | 6 | 5 | 6 | 5 | 5 | 5 | 5 |
| Minimum | 921 | 27.2 | 7.62 | .264 | .164 | 1.045 | 1.316 | .567 | 1.465 | .500 | 6.80 |
| Maximum | 1001 | 33.3 | 8.10 | .281 | .184 | 1.094 | 1.422 | .682 | 1.706 | .606 | 8.04 |
| Mean | 966 | 30.8 | 7.87 | .270 | .174 | 1.075 | 1.371 | .635 | 1.546 | .544 | 7.23 |
| **Composite F** | | | | | | | | | | | |
| Samples [3] | 11 | 11 | 11 | 11 | 11 | 11 | 11 | 11 | 11 | 11 | ... |
| Minimum | 1341 | 31.9 | 9.62 | .318 | .219 | 1.860 | 2.192 | 0.921 | 2.327 | .664 | ... |
| Maximum | 1573 | 41.4 | 10.79 | .375 | .242 | 2.003 | 2.355 | 1.036 | 3.085 | .845 | ... |
| Mean | 1433 | 35.5 | 10.31 | .347 | .229 | 1.927 | 2.286 | 0.979 | 2.568 | .771 | ... |
| SD | 78.1 | 2.7 | 0.31 | .024 | .008 | 0.060 | 0.052 | 0.034 | 0.223 | .051 | ... |
| SE$_m$ | 23.6 | 0.8 | 0.09 | .007 | .002 | 0.018 | 0.016 | 0.010 | 0.067 | .015 | ... |

[1] Amounts of the individual foods in each composite are given in table 4.

[2] Heat of combustion.

[3] Triplicate analyses were obtained for each sample.

were comparable. Composite A, however, had the highest accuracy with respect to calcium and phosphorús analyses. The fact that this mixture was the only one which contained milk, and thus possessed relatively greater quantities of these two elements, undoubtedly helps to explain the greater accuracy of these determinations. The number of samples analyzed increases the accuracy of all the mean values; since, as the number of samples analyzed is increased, the range of variation of the constituent foods is more nearly included, and a true mean figure approached. This is supported by the results obtained for composite A, which represents analyses of twenty separate diets, and D, which is based on seventeen analyses of composites. Composite D had the smallest standard error of the mean for nitrogen, chlorine, sulfur and fat.

### SUMMARY

1. The analyses of twenty-two common foods for nitrogen, fat, energy, the positive (calcium, magnesium, sodium and potassium) and negative (phosphorus, chlorine and sulfur) minerals and iron indicate that individual samples of a given food vary from values reported in the standard tables.

2. Fruits and vegetables vary widely while milk is more constant in mineral content.

3. The variability of common foods does not seem to be as much a measure of contamination as a determination of real differences in composition.

4. Comparison of a series of analyses of eleven composite diets with the sums of the corresponding values for the individual foods in the diet, emphasizes the increased accuracy which may be obtained when a larger amount of a given constituent is contained in the material available for analysis. The composite diets showed a more constant composition than the components.

5. When the analyses of composite diets are compared with dietary figures calculated from the literature, there is good agreement in the content of magnesium, potassium, phosphorus, sulfur, calories and fat. Sodium, chlorine and calcium may be significantly different from calculated values.

6. If mineral, energy or fat content of a composite diet or an individual food is to be known with the highest degree of accuracy, it should be analyzed under the conditions of the experiment in which it is to be used.

7. The variations from time to time, as indicated by the standard error of the mean of each series, did not vary appreciably for a given constituent excepting in the case of sodium and chloride, even when the technique was refined in every possible manner. This indicates that the variations are inherent in the foods themselves rather than measures of errors in manipulation.

## LITERATURE CITED

BASSETT, SAMUEL H., AND HELEN E. VAN ALSTINE 1935 Mineral exchanges of man. IV. Variations in the mineral content of diets with a constant raw weight formula. J. Nutrition, vol. 9, p. 175.

BISHOP, EDNA R. 1934 The calcium and phosphorus content of some Alabama vegetables. J. Nutrition, vol. 8, p. 239.

BUTLER, A. M., AND ELIZABETH TUTHILL 1931 An application of the uranyl zinc acetate method for determination of sodium in biological material. J. Biol. Chem., vol. 93, p. 171.

COLEMAN, J. M., AND R. W. RUPRECHT 1935 The effect of fertilizers and soil types on the mineral composition of vegetables. J. Nutrition, vol. 9, p. 51.

DAVIDSON, JEHIEL, AND J. A. LeCLERC 1936 The variation in the mineral content of vegetables. J. Nutrition, vol. 11, p. 55.

FLOSDORF, EARL W., AND STUART MUDD 1938 An improved procedure and apparatus for the preservation of sera, micro-organisms and other substances. The cryochem process. J. Immunology, vol. 34, p. 469.

GARELLI, F. 1932 The determination of sulfur, chloride and bromine in organic products by means of the calorimetric bomb. Chimie et Industrie, vol. 27, p. 166.

GREAVES, J. E., AND C. T. HIRST 1929 The mineral content of grain. J. Nutrition, vol. 1, p. 293.

GUTMAN, ALEXANDER B., AND MARGARET LOW 1939 On the approximation of the calculated to determined calcium content of human dietaries. J. Nutrition, vol. 18, p. 257.

HAWKS, JEAN E., MARIE DYE AND MERLE M. BRAY 1937 An improved technic for metabolism studies in pre-school children with a statistical determination of its reliability. J. Nutrition, vol. 13, p. 51.

HAWKS, JEAN E., MERLE M. BRAY, JEANNE M. VOORHEES, BARBARA VELEY, CHRISTINE CARLSON AND MARIE DYE 1940 The influence of technic on the composition values of identical diets. J. Am. Diet. Assoc., vol. 16, p. 416.

HUMMEL, FRANCES COPE, AND H. H. WILLARD 1938 Determination of iron in biological materials. The use of o-phenanthroline. Indust. Eng. Chem., Analyt. Ed., vol. 10, p. 13.

HUMMEL, FRANCES C., MARION L. SHEPHERD AND ICIE G. MACY 1940 Effect of changes in food intakes upon the lignin, cellulose, and hemicellulose contents of diets. J. Am. Diet. Assoc., vol. 16, p. 199.

KRAMER, BENJAMIN, AND FREDERICK TISDALL 1921 A clinical method for the quantitative determination of potassium in small amounts of serum. J. Biol. Chem., vol. 46, p. 339.

McCANCE, R. A., AND R. D. LAWRENCE 1929 Carbohydrate content of foods. Med. Res. Council, Special Report Series, no. 135.

McCANCE, R. A., E. M. WIDDOWSON AND L. R. B. SHACKLETON 1936 The nutritive value of fruits, vegetables and nuts. Med. Res. Council, Special Report Series, no. 213.

MACKAY, E. M., AND A. M. BUTLER 1935 A comparison of the mineral (base) composition of dietaries as determined from tables and by analyses. J. Am. Diet. Assoc., vol. 10, p. 398.

MACY, ICIE G. 1942 Nutrition and chemical growth in childhood. Vol. I. Evaluation. Charles C. Thomas, Springfield, Ill.

PETERS, JOHN P., AND DONALD D. VAN SLYKE 1932 Quantitative clinical chemistry. Vol. II. Methods. Williams and Wilkins Co., Baltimore, p. 859.

SCALES, F. M., AND A. P. HARRISON 1920 Boric acid modification of the Kjeldahl method for crop and soil analysis. Indust. Eng. Chem., vol. 12, p. 350.

SCHUMPF-PIERRON, P. 1932 Varietal differences in mineral equipment of cereals. C. R. Soc. Biol., vol. 111, p. 846.

SCOTT, WILFRED W. 1939 Standard methods of chemical analysis. Vol. I. The elements. 5th ed., D. Van Nostrand Co., Inc., New York.

SHERMAN, HENRY C. 1941 Chemistry of food and nutrition. 6th ed., The Macmillan Co., New York.

SNEDECOR, GEORGE W. 1934 Calculation and interpretation of analysis of variance and covariance. Collegiate Press, Inc., Ames, Iowa.

TEAGUE, D. MAXWELL, HARRY GALBRAITH, FRANCES COPE HUMMEL, HAROLD H. WILLIAMS AND ICIE G. MACY 1942 Effects of dessication procedures on the chemical composition of feces, urine, and milk. J. Lab. Clin. Med. (in press).

WILLARD, HOBART H., AND N. HOWELL FURMAN 1935 Elementary quantitative analysis. 2nd ed., D. Van Nostrand Co., Inc., New York.

WILLIAMS, RAY D., AND W. H. OLMSTED 1935 A biochemical method for determining indigestible residue (crude fiber) in feces: lignin, cellulose and non-water soluble hemicellulose. J. Biol. Chem., vol. 108, p. 653.

WORD, AUBREY H., AND GLEN WAKEHAN 1938 Variations in mineral food analyses. University of Colorado Studies, vol. 25, p. 181.

# THE UTILIZATION OF CAROTENE AND VITAMIN A
## .IN THE RAT

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Attention in recent years has been directed towards those factors influencing the availability of carotene and vitamin A. The method generally conceded to be the most suitable for such studies is based upon the determination of the vitamin in the livers of rats previously fed equivalent amounts of carotene or vitamin A from different sources; the conversion of carotene or the vitamin into liver stores of the latter when fed in excess of the daily requirement being made the measure of utilization.

The results of previous investigations have shown conclusively that the principal storage center for vitamin A in the rat is the liver (Sherman and Boynton, '25; Moore, '31; and Baumann, Riising and Steenbock, '34).

That a relationship between the availability of dietary vitamin A potency and the liver storage of vitamin A is valid, has been amply demonstrated by Davies and Moore ('34, '35), Greaves and Schmidt ('35), Lease, Lease, Steenbock and Baumann ('39), Gray, Hickman and Brown ('40), and Smith and Otis ('41).

It becomes increasingly important that further information be obtained on those factors that might affect the significance of results obtained from studies on the storage of vitamin A in the liver and the relative availability of carotene and vitamin A for this purpose under controlled experimental conditions. It was with these objects in view that the herein-reported experiments were carried out.

[1] Now at the Patuxent Research Refuge, Bowie, Maryland.

## EXPERIMENTAL

The storage of vitamin A in the rat livers was measured by means of a Bausch and Lomb medium quartz spectrograph by a method already described by Fraps and Kemmerer ('38).

In all the experiments male rats were used, and each replicate contained litter mates of approximately the same weight. Distilled water was given and, except where otherwise noted, diet 678-B (table 1) which is vitamin A-free. In all cases care

TABLE 1

*Ingredients of diets used.*

|  | 678-B | 7027-A |
|---|---|---|
|  | % | % |
| Heated cornstarch [1] | 59.0 |  |
| Heated casein [1] | 22.0 |  |
| Irradiated yeast | 10.0 | 0.53 |
| Salt mixture no. 7009 | 4.0 |  |
| Sodium chloride | 1.0 | 0.50 |
| Wesson oil | 4.0 | 3.9 |
| Cornmeal (white) |  | 49.5 |
| Cottonseed meal |  | 9.7 |
| Powdered skim milk |  | 29.1 |
| Wheat bran |  | 1.9 |
| Non-irradiated yeast |  | 4.3 |
| Calcium citrate |  | 0.5 |
| Ferric chloride |  | 0.05 |
| Copper sulphate |  | 0.02 |

[1] Cornstarch and casein heated at a temperature of 110° C., 24 hours.

was taken to equalize the food intake between litter mates. In order to eliminate food wastage the diet was moistened to a paste-like consistency prior to feeding.

In the first experiment a comparison was made of the vitamin A storage in the livers of rats that received equivalent amounts of vitamin A potency, as found in cod liver oil, carotene dissolved in cottonseed oil, and carotene as contained in alfalfa leaf meal. Four groups of rats containing eight replicates were used. The animals were placed on the experiment when 28 days old, the time they were weaned. From the time the young were born until weaning, the females received, ad

libitum, the regular stock diet for nursing females (diet 7027-A, table 1), which has a very low vitamin A potency.

The rats in group 1, the negative controls, received daily 0.1 ml. of cottonseed oil as a supplement. Rats in group 2 received daily 0.1 ml. of cottonseed oil containing 60 I. U. of vitamin A potency in the form of 36 µg. of carotene purified by precipitation with methyl alcohol (Fraps and Kemmerer, '39). The rats in group 3 received daily enough alfalfa leaf meal of high carotene content to give 60 I. U. of vitamin A potency as determined by the method of Fraps and Kemmerer ('39). The rats in group 4 received daily 60 I. U. of vitamin A from U. S. P. XI reference cod liver oil, dissolved in 0.1 ml. of cottonseed oil. All rats were on experiment for 14 days, then killed, their livers removed, and the vitamin A content determined spectrographically for each liver. In all the experiments, two or more litter mates were killed at the time of weaning, and the vitamin A content of their livers determined. All results are expressed in total micrograms of spectro vitamin A found in each liver.

In the second experiment, designed to show the effect of time of depletion on the vitamin A stored in the weaning rat's liver, the same general methods of selection and analysis were followed, except that two groups of eight replicates were used. The rats in group 1 were on experiment 2 weeks, and those in group 2 for 4 weeks. All rats received the basal diet (678-B), food intakes being equalized between litter mates; the same level of food intake was used for the surviving rat in the replicate during the last 2 weeks of this experiment.

In the first and second experiments it was observed that an increase occurred in the spectro vitamin A content of the livers even when the rat received a diet devoid of this vitamin. The third experiment was designed to show the causes of this increase. Three groups of rats consisting of eight replicates were placed on experiment when they were 21 days old. The feeding period was 7 days. The rats in group 1 received diet 678-B; the rats in group 2 received a similar diet but with the yeast replaced with an equal weight of cornstarch. Thia-

mine hydrochloride was fed daily to the rats in group 2 in sufficient amount to cover their daily thiamine requirements. The rats in group 3 were fed the stock diet (7027-A). The data obtained from all the experiments were analyzed statistically by the method of analysis of variance as given by Snedecor ('38).

### RESULTS

The data obtained from the first experiment are presented in table 2. An increased retention of vitamin A is found in all diet groups over the values for the controls at the beginning. Those rats receiving vitamin A showed greatest retention of the vitamin, the animals receiving carotene in

TABLE 2

*Effect of different sources of vitamin A potency on the spectro vitamin A content of rat livers.*

| | NO. OF RATS | SPECTRO VITAMIN A PER LIVER, IN MICROGRAMS | | |
| | | Range | Mean | Standard deviation of mean |
|---|---|---|---|---|
| Contents at beginning | 25 | 2.5– 18.2 | 8.2 | 3.7 |
| Contents at end | 9 | 21 – 39 | 29.7 | 5.8 |
| Carotene in cottonseed oil [1] (36 μg.) | 9 | 42 – 88 | 67.0 | 15.4 |
| Carotene in alfalfa (36 μg.) | 9 | 29 – 63 | 42.7 | 12.0 |
| Vitamin A in cottonseed oil [1] (60 I.U.) | 9 | 70 –121 | 92.9 | 17.0 |

Difference between litters is significant; difference between diet groups is significant.

[1] Wesson.

cottonseed oil gave a lower retention, and those fed carotene in alfalfa leaf meal gave the lowest retention. The differences between diet groups and between replicates are statistically significant. Assuming the efficiency of vitamin A from cod liver oil to be 100% for liver storage purposes, carotene in cottonseed oil was found to be 59% as efficient, and carotene as found in alfalfa leaf meal was 21% as efficient at the potency level fed. Carotene as found in alfalfa leaf meal was 35% as efficient for vitamin A storage purposes as was carotene

dissolved in cottonseed oil. No account was taken of the possible retention of the carotene in the liver as such, since previous observations have shown that such retentions at levels similar to those fed are so slight as to be negligible.

It is observed that the negative controls also showed an increase in the vitamin A content of the liver over that found in the litter mates killed at the start of the experimental feeding; it is apparent that some substance must have been present in the vitamin A free diet (678-B) that is retained in the liver and gives a false vitamin A value upon spectrographic analysis. However, the relative retentions remain valid with the type of experimental design used for these studies.

TABLE 3

*Effect of length of time of depletion on the spectro vitamin A content of rat livers.*

| | NO. OF RATS | SPECTRO VITAMIN A PER LIVER, IN MICROGRAMS | | |
| | | Range | Mean | Standard deviation of mean |
|---|---|---|---|---|
| At beginning | 13 | 8.4–30.8 | 15.1 | 6.4 |
| Depleted 2 weeks | 7 | 19.4–27.5 | 24.3 | 3.2 |
| Depleted 4 weeks | 7 | 20.9–31.9 | 26.1 | 3.9 |

Difference between beginning and second week is significant;
difference between second and third week is not significant.

The data presented in table 3 summarize the results obtained in the second experiment. A definite increase is observed in the liver stores of all rats maintained on the vitamin A free diet (678-B) for a period of 2 weeks or longer; this increase is significant. No significant increases occurred in the liver store of vitamin A in those rats continued on diet 678-B for an additional 2 weeks, as compared with their litter mates killed at the end of the first 2-week period. Whether the liver reached a saturation point for the substance giving the false vitamin A value is not known; however, it is known that animals depleted for a 28-day period exhibit symptoms of vitamin A deficiency, and it is not in accord with such observations

to find vitamin A liver stores of such magnitude in depleted rats.

In the third experiment designed to determine the cause of the increase in spectro vitamin A in the livers of the rats maintained on a vitamin A free diet for a period of 2 weeks, yeast was selected as the component of the diet most likely to be responsible. It was found upon spectrographic analysis that yeast did contain a substance capable of giving a false vitamin A value. Other substances were found by Fraps and Kemmerer ('38) to give false vitamin A values, and such substances are referred to as pseudo vitamin A.

Data obtained from the third experiment are presented in table 4. Those rats receiving diet 678-B which had the yeast

TABLE 4

*Effect of diet low in vitamin A potency upon spectro vitamin A in rat livers.*

| | NO OF RATS | SPECTRO VITAMIN A PER LIVER, IN MICROGRAMS | | |
| | | Range | Mean | Standard deviation of mean |
|---|---|---|---|---|
| Content at beginning | 16 | 3.1– 8.5 | 5.8 | 1.6 |
| Basal diet without yeast | 8 | 8.4–13.3 | 10.5 | 2.0 |
| Basal diet including yeast | 8 | 9.9–19.4 | 15.4 | 3.5 |
| Starch ration | 8 | 10.9–15.1 | 13.4 | 1.9 |

Differences between litters is significant; difference between diet groups is significant.

replaced with cornstarch showed the least increase of pseudo vitamin A in the liver. The rats in the group receiving diet 7027-A which contained a small percentage of yeast had a significantly greater amount of pseudo vitamin A, while the group of rats receiving the regular basal diet (678-B) which is vitamin A free but contains the greatest amount of yeast, showed the greatest storage of pseudo vitamin A. All differences between diet groups are significant, and it is quite evident that the yeast was responsible for a considerable portion of the increase in the liver stores of pseudo vitamin A. Further examination would probably show that other com-

ponents of the diet were also contributing to the increase of the liver stores. It is obvious that equalized food intake is necessary in all cases where direct comparisons are made of the relative availability of different sources of vitamin A potency using the liver stores of vitamin A as a basis for the evaluation.

## SUMMARY AND CONCLUSIONS

Using paired feeding technique and spectrographic analytical methods, investigations have been made on the utilization of vitamin A and carotene for liver storage of this vitamin in rats.

Vitamin A in cod liver oil was found to be most efficient for building up liver stores, carotene dissolved in cottonseed oil was 59% as effective, and carotene as found in alfalfa leaf meal was 21% as effective.

Carotene in alfalfa leaf meal was found to be 35% as efficient as carotene dissolved in cottonseed oil for building up the rat's liver store of vitamin A.

The spectro vitamin A content of the liver was found to increase significantly during the 2 weeks following weaning despite the fact that the experimental rats received a vitamin A free diet. No further significant increase occurred in the livers of litter mates that were continued on the same diet for an additional 2 weeks. Yeast present in the diet was found to contribute significantly to this increase.

The importance of equalizing food intakes and using litter mates in experiments involving the relative utilization of vitamin A and carotene, is stressed particularly when chemical and physical quantitative analytical procedures are employed in conjunction with the animal experimentation.

## LITERATURE CITED

BAUMANN, C. A., B. M. RIISING AND H. STEENBOCK 1934 Fat soluble vitamins. XLII. The absorption and storage of vitamin A in the rat. J. Biol. Chem., vol. 107, p. 705.

DAVIES, A. W., AND T. MOORE 1934 Vitamin A and carotene. XI. The distribution of vitamin A in the organs of the normal and hypervitaminotic rat. Biochem. J., vol. 28, p. 288.

DAVIES, A. W., AND T. MOORE 1935 Vitamin A and carotene. XII. The elimination of vitamin A from livers of rats previously given massive doses of vitamin A concentrate. Biochem. J., vol. 29, p. 147.

FRAPS, G. S., AND A. R. KEMMERER 1938 The relation of the spectro-vitamin A and carotene content of butter to its vitamin A potency measured by biological methods. Texas Agr. Exp. Sta. Bull. 560.

———————— 1939 Improved method for estimating carotene in feeds. J. Assoc. Off. Agr. Chem. 22, p. 190.

GRAY, E. LE B., K. C. D. HICKMAN AND E. F. BROWN 1940 The state of vitamin A in the liver of the rat after feeding various forms of the vitamin. J. Nutrition, vol. 19, p. 39.

GREAVES, J. D., AND C. L. A. SCHMIDT 1935 On the absorption and utilization of carotene and vitamin A in choledocholonostomized vitamin A deficient rats. Am. J. Physiol., vol. 111, p. 492.

LEASE, E. J., J. G. LEASE, H. STEENBOCK AND C. A. BAUMANN 1939 The biological value of carotene in various fats. J. Nutrition, vol. 17, p. 91.

MOORE, T. 1931 Vitamin A and carotene. VIII. The distribution of vitamin A and carotene in the body of the rat. Biochem. J., vol. 25, p. 275.

SHERMAN, H. C., AND L. C. BOYNTON 1925 Quantitative experiments upon the occurrence and distribution of vitamin A in the body and the influence of the food. J. Am. Chem. Soc., vol. 47, p. 1646.

SMITH, M. C., AND L. OTIS 1941 Observations on carotene analysis of vegetables and fruits as a basis for prediction of their vitamin A value. Food Res., vol. 6, p. 143.

SNEDECOR, G. W. 1938 Statistical methods applied to experiments in agriculture and biology. Collegiate Press, Inc., Ames, Iowa.

# A LIVER CONCENTRATE AS A SOURCE OF UNRECOGNIZED VITAMINS REQUIRED BY THE CHICK [1]

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ONE FIGURE

It was shown by Hogan and Boucher ('33) that liver extracts are an excellent source of the unrecognized vitamins required by the chick. Simplified diets which contain these extracts promote a rapid rate of growth and prevent abnormalities often encountered in chicks on this type of ration. According to Hogan and Richardson ('40) three fractions of dried beef liver were required for the maximum rate of growth. These were (1) a 95% alcohol extract, (2) a water extract which followed the alcohol extraction, and (3) either the residue which followed the alcohol and water extractions or the acid-hydrolyzed residue. Although the separation was not complete, the evidence seemed to indicate that each fraction contained at least one essential nutrient which was more concentrated in it than in the other two.

Many new vitamins are now available in pure form, and it is possible to use less complicated rations than those formerly employed. The object of this report is to describe the degree of success obtained in substituting synthetic vitamins for the crude vitamin carriers formerly employed.

## EXPERIMENTAL

The experimental procedure was the same as that previously described (Hogan and Richardson, '40). The chicks were approximately 1 day old when delivered from the hatchery, and they were supplied with the experimental ration immediately. The composition of the basal ration, which is similar to those described in earlier publications, is given in table 1.

TABLE 1

*Composition of basal ration.*

| | |
|---|---|
| Casein ................. ........... | 35 |
| Starch .. ..... ........... ...... | 31 |
| Lard ........................... | 17 |
| Salts (Hubbell et al. [ '37] with $MnSO_4$ added to make 0.025 Mn in the ration) .................... | 4 |
| Cellulose ....... .... ...... ..... | 3 |
| Gelatin ......... ........... ..... | 10 |

Vitamins [1] per 100 gm. of ration

| | |
|---|---|
| Vitamin A percomorph liver oil...... | 6000 I. U. |
| Vitamin D ....................... | 850 I. U. |
| Thiamine [2] .......... ......... | 0.8 mg. |
| Riboflavin [3] ......... ........ ... | 1.6 mg. |
| Pyridoxine [3] ..................... | 1.2 mg. |
| Calcium pantothenate [2] ......... . | 2.0 mg. |
| Choline [3] .............. ... ... ... | 400.0 mg. |
| Inositol .............. ...... .. | 100.0 mg. |
| p-aminobenzoic acid .. ... . ..... | 30.0 mg. |
| Nicotinic acid [3] .................. | 1.0 mg. |
| 2 methyl-1,4-naphthoquinone [2] ... ... | 1.0 mg. |
| Alpha tocopherol [3] ..... ...... .... | 8.0 mg. |
| Biotin [3] ......................... | 2.0 $\mu$g. per chick per day |

The crude vitamin carriers were substituted on a dry matter basis for an equal weight of starch.

[1] This ration contains large quantities of vitamins and in some instances it may contain more than is required, but there was never any indication of toxicity. In general the results were more uniform when the vitamins were supplied in large quantity than when they were supplied in smaller amounts. The vitamins were added to the ration as solutions during the process of mixing to insure even distribution.

[2] Generously supplied by Merck and Co., Rahway, New Jersey.

[3] Concentrate no. 200, S. M. A. Corporation, Chicago, Illinois.

*Preparation of the water extract of liver.* In the earlier experiments the liver fractions were prepared by the method previously described by Hogan, Richardson, Patrick and Kempster ('41). It is now possible to eliminate both the alcohol extract and the acid-hydrolyzed liver residue. The water extract which is still indispensable may be prepared by a simpler method. In this new procedure fresh beef liver is extracted with boiling water. The water extract is concentrated to a convenient volume, and a large portion of the inactive material is removed by the addition of 95% alcohol to yield a concentration of 50%. This extract is concentrated to a thick paste and is designated the water extract of liver (WE). It contains all the unrecognized vitamins required by the chick, unless some unsuspected ones are concealed in the other organic constituents of the diet. The active agent is adsorbed from the water extract on fuller's earth at a pH of 1.0 (FEA), and is then eluted from the adsorbate with 0.2 N ammonia (FEE). A condensed description of these procedures is given in table 2.

*The alcohol extract is dispensable.* Since it was stated in earlier reports from this laboratory that the 95% alcohol extract is indispensable, our present view that it is not essential needs some explanation. This fraction supplied pyridoxine and pantothenic acid, both of which are required by the chick and are now supplied in the ration in the pure form. In addition to these two vitamins this fraction supplied a specific organic antiperotic substance (Hogan, Richardson and Patrick, '40) which has been identified as choline (Jukes, '40 a, '40 b; Hogan et al., '41; Hegsted, Mills, Elvehjem and Hart, '41). The alcohol extract, therefore, is not essential when the ration contains 0.1% of choline in addition to the other vitamins which are now available in pure form. Larger amounts of choline do not improve the ration.

*Gelatin may be substituted for the residue fraction.* In view of the reports that glycine and other compounds are essential for the growth of chicks (Almquist and Mecchi, '40;

Almquist, Stokstad, Mecchi and Manning, '40 a, '40 b; Stokstad, Almquist, Mecchi, Manning and Rogers, '41; and Hegsted, Briggs, Elvehjem and Hart, '41), gelatin was substituted for the acid-hydrolyzed liver residue. When all the recognized vitamins were supplied, the rate of growth was as rapid with gelatin as with the hydrolyzed material. There

TABLE 2

*Scheme followed in preparation of various fractions.*

100 pounds of ground fresh beef liver extracted 3 times with 12 gallons of boiling water for each extraction. The first 12 gallons were brought to a pH of 5.0 with $H_2SO_4$

Residue (R)

Extracts combined, concentrated in vacuo to 12 liters. 95% ethyl alcohol added to a concentration of 50%

Filtrate (WE), concentrated in vacuo until free from alcohol, diluted until 1 liter contains 100 gm. dry matter. Adsorbed 4 times at pH of 1.0 with 20 gm. of fuller's earth for each adsorption. The pH was adjusted with $H_2SO_4$

Alcohol precipitate (AP)

Adsorbates combined (FEA). Eluted 3 times, with 600 cc. of 0.2 N $NH_3$ per 100 gm. of adsorbate for each elution

Filtrate. Excess sulphate removed with $Ba(OH)_2$

Eluate, concentrated in vacuo (FEE)

Residue discarded

$BaSO_4$ discarded

Filtrate, water removed in vacuo (FEF)

is no certainty that gelatin is free from contamination with vitamins and it would be preferable to omit it, but at present it is decidedly useful. It is probably preferable to the acid-hydrolyzed residue, as it is presumably less complex.

*Unrecognized vitamins of the water extract of liver.* In our earlier studies all attempts to dispense with a water extract of liver, such as preparation 4080, had ended in complete

failure, but at that time only a small proportion of the vitamins now recognized had been isolated, and were available. It seemed desirable, therefore, to reinvestigate the nutritional properties of the water extract to determine whether or not this fraction is still indispensable. The data are summarized in figure 1. Group IV received the basal diet and grew very slowly. In addition this group developed a perosis that is not due to a deficiency of either manganese or choline (Richardson and Hogan, '41). Group III received a fuller's earth eluate of the water extract, but biotin was omitted from the vitamin mixture. The growth rate was improved, but every chick developed dermatitis on the feet as described by Hegsted, Oleson, Mills, Elvehjem and Hart ('40). Group II received both the eluate and biotin. The growth rate was normal and dermatitis did not develop. Group I received the water extract itself, but the chicks did not grow any more rapidly than did those in group II. Since the vitamin mixture of group I did not contain biotin it is clear that this vitamin is present in the water extract.

The data in figure 1 show that the water extract of liver contains nutrients, in addition to the recognized vitamins, that are required for normal development of the chick. About 60% of the total activity of the original water extract was recovered in the fuller's earth eluate, and on the basis of equivalent amounts of dry matter, the active agent is concentrated approximately tenfold by this procedure. The remainder of the activity was lost, apparently due to destruction while in contact with acid during the adsorption process. Attempts to reduce this loss by adsorption in less acid solutions did not improve the procedure, because the amount of the active agent that was adsorbed decreased as the pH of the solution was increased. Only a small amount was adsorbed at a pH of 5.5. Our data indicate that the eluate is fully equal to the original water extract as a source of the unrecognized vitamins required by the chick.
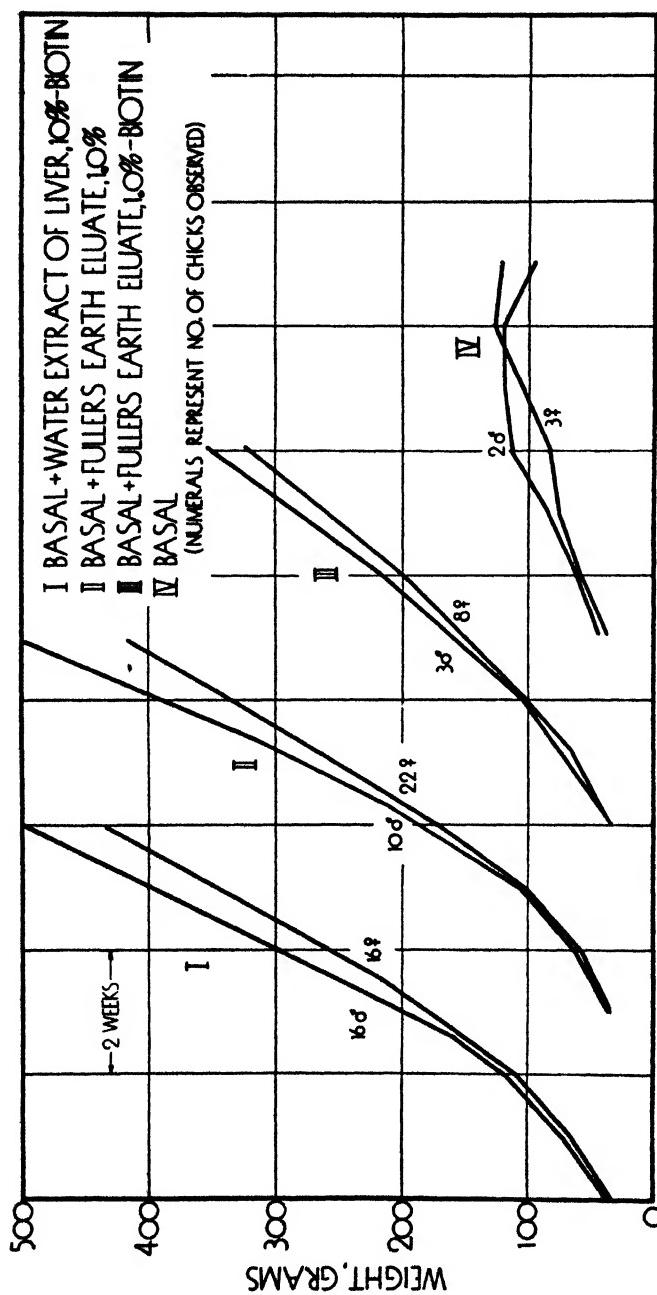
Fig. 1 The rate of growth on the basal ration (IV) is unsatisfactory, and most of the chicks developed perosis. When the fuller's earth eluate is added to the basal diet (II) the growth rate is normal, but when biotin is omitted from the vitamin mixture (III) dermatitis develops and the growth rate is retarded. Biotin may be omitted when the water extract (I) is supplied. The water extract evidently supplies this vitamin.

## SUMMARY

1. When chicks receive a ration that contains no vitamins except those now recognized as such, they grow slowly and develop perosis.

2. A water extract of beef liver contains all unrecognized vitamins required by the chick.

3. The unrecognized vitamins required by chicks are adsorbed from a water extract of liver by fuller's earth at a pH of 1.0. The activity is readily eluted by 0.2 N ammonia. During the adsorption procedure approximately one-third of the total activity is lost.

4. A simplified diet has been prepared that is adequate for the growth of chicks and does not contain over 1% of crude vitamin carriers.

## LITERATURE CITED

ALMQUIST, H. J., AND E. MECCHI 1940 Identification of the rice factor. The essential nature of the glycine component. J. Biol. Chem., vol. 135, pp. 355–356.

ALMQUIST, H. J., E. L. R. STOKSTAD, E. MECCHI AND P. D. V. MANNING 1940 a Identification of the rice factor. J. Biol. Chem., vol. 134, pp. 213–216.

———— 1940 b Identification of the rice factor. The carbohydrate component. J. Biol. Chem., vol. 134, pp. 465–466.

HEGSTED, D. M., G. M. BRIGGS, C. A. ELVEHJEM AND E. B. HART 1941 The role of arginine and glycine in chick nutrition. J. Biol. Chem., vol. 140, pp. 191–199.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., vol. 138, pp. 459–466.

HEGSTED, D. M., J. J. OLESON, R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1940 Studies on a dermatitis in chicks distinct from pantothenic acid deficiency. J. Nutrition, vol. 20, pp. 599–606.

HOGAN, A. G., AND R. V. BOUCHER 1933 The nutritional requirements of the chick. Mo. Agr. Exp. Sta. Res. Bul. 198.

HOGAN, A. G., AND L. R. RICHARDSON 1940 Simplified rations for the chick. J. Nutrition, vol. 19, pp. 1–11.

HOGAN, A. G., L. R. RICHARDSON AND H. PATRICK 1940 Relation of perosis to unrecognized vitamins. J. Nutrition, vol. 19, Proc., p. 14.

HOGAN, A. G., L. R. RICHARDSON, H. PATRICK AND H. L. KEMPSTER 1941 Perosis due to a vitamin deficiency. J. Nutrition, vol. 21, pp. 327–340.

HUBBELL, REBECCA B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, pp. 273–285.

JUKES, THOMAS H. 1940 a  Prevention of perosis by choline. J. Biol. Chem., vol. 134, pp. 789–790.

————— 1940 b  Effect of choline and other supplements on perosis. J. Nutrition, vol. 20, pp. 445–458.

RICHARDSON, L. R., AND A. G. HOGAN  1941  The antiperosis complex. Proc. Soc. Exp. Biol. and Med., vol. 48, pp. 459–461.

STOKSTAD, E. L. R., H. J. ALMQUIST, E. MECCHI, P. D. V. MANNING AND R. E. ROGERS  1941  The carbohydrate component of the rice factor. J. Biol. Chem., vol. 137, pp. 373–375.

# POLYNEUROPATHY IN THIAMINE DEFICIENT RATS DELAYED BY ALCOHOL OR WHISKY [1]

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FOUR FIGURES

## INTRODUCTION

In 1934 Cowgill proposed a formula relating the vitamin B requirement to body weight and caloric intake. This relationship was used by Jolliffe and co-workers (Jolliffe, Colbert and Joffe, '36; Jolliffe and Colbert, '36) in their calculations to show that a deficiency of vitamin B ($B_1$) was present in the diets of alcoholic, polyneuritic patients. The occurrence of polyneuritis could not be predicted consistently unless the calories from alcohol were included with the diet calories. Laboratory investigations (Evans and Lepkovsky, '29, '34, '35; Kemmerer and Steenbock, '33; McHenry, '37; Salmon and Goodman, '37) have shown that the vitamin $B_1$ requirement is influenced by the composition of the diet as well as by the total number of calories. An abundant carbohydrate intake increases the need for thiamine while the substitution of fat for carbohydrate lessens the need and delays the onset of polyneuropathy. Since there was no evidence to the contrary, the assumption has been retained that the metabolism of alcohol increased the need for thiamine, and on this basis alcohol has been indicted as a more or less specific factor in producing the polyneuropathy seen in alcoholic patients.

[1] Read at the Boston meeting of the American Institute of Nutrition, April 1, 1942.

Because of the lack of direct evidence on the relationship of alcohol intake to the thiamine need, an experimental investigation was undertaken.

## EXPERIMENTAL

Albino rats at weaning (22 days old) were used throughout the experiment; paired rats were litter mates of the same sex. The rats were housed in individual, elevated, wide-mesh galvanized wire cages. Two strains of rats were used, one in the experiments with whisky and another in the alcohol experiments. The ethyl alcohol used was approximately 20% by volume and the whisky was a 100 proof commercial brand diluted with an equal volume of water.

All the animals were fed diet no. 461 which had the following percentage composition: leached and alcohol extracted casein, 18; sucrose, 73; cottonseed oil,[2] 3; cod liver oil, 2; Osborne and Mendel salt mixture, 4. A daily supplement of 50 µg. of riboflavin, 50 µg. of calcium pantothenate, 20 µg. of pyridoxine, 1 mg. of nicotinic acid, and 2 mg. of choline was given to each rat in a supplement dish. For the first 42 days 4 µg. of thiamine chloride were added to the daily vitamin supplement. The number of days from the complete withdrawal of the thiamine to the onset of acute polyneuropathy was recorded. Polyneuropathy was considered to be present when the rat was spastic, and had an ataxic, tonic convulsive seizure when dropped on its back from a height of 3 to 5 inches. Death occurred in untreated rats within 48 hours of the time that this syndrome was first observed.

### Experiment I

#### Effect of whisky with isocaloric intake

Twelve pairs of litter mates were used for this experiment. One rat in each pair drank whisky ad libitum, and the other, water ad libitum. The two rats of each pair received an

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[2] Wesson.

isocaloric intake throughout the experiment (based on 4.1 calories per gram for the food and 1.5 calories per cubic centimeter for the diluted whisky).

The whisky intake was recorded by means of an especially designed apparatus which collected the whisky wasted by the animal. A 10% allowance was made for evaporation based on losses from a similar apparatus set up in an adjoining empty cage. The apparatus was filled with a measured quantity of whisky. The collected waste and the remainder were measured and the dietary caloric equivalent of the consumed whisky was calculated. The amount of food offered to the two rats of each pair was adjusted daily to equalize the caloric intake according to the calculated average daily intake of whisky. After the development of polyneuropathy in either of the paired rats, the remaining animal was fed diet no. 461 ad libitum.

Because of the calories supplied by the whisky the average intake of diet no. 461 for the rats receiving whisky was 74 to 82% (average 79%) of the amount of this diet eaten by the rats getting water. The average daily intake of diet no. 461 up to the time of development of polyneuropathy (a) of the rats receiving whisky was 3.9 gm., and (b) of the rats receiving water, 5.0 gm.

*Results.* In every one of the twelve pairs of rats polyneuropathy first occurred in the rat that drank water (fig. 1). In the group as a whole the average time between the withdrawal of thiamine chloride and the onset of polyneuropathy in the rats on water was 18 days; in the rats on whisky, 33 days (an 83% longer interval). During the 42-day preliminary period in eleven of twelve pairs the rats receiving whisky had a gain in body weight which was less (average 23%) than that of the litter mates receiving water. In one pair the rat that drank water gained less (9%) than the rat receiving whisky. A rapid decline in body weight occurred in both the rats getting whisky and those receiving water following the complete withdrawal of the thiamine.

**EFFECT OF WHISKY WITH ISOCALORIC INTAKE**
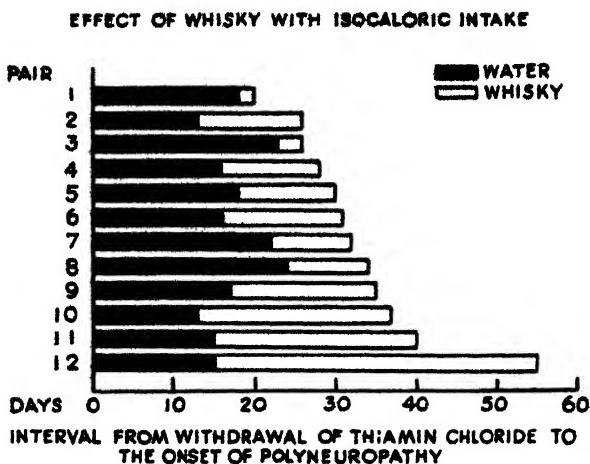


Figure 1

## Experiment II

### Effect of whisky with equal food intake

Twelve pairs of litter mates were used for this experiment. The rats of each pair were allowed equal amounts of diet no. 461 until one developed polyneuropathy. The paired rat was then fed diet no. 461 ad libitum. Throughout the experiment one rat in each pair was given diluted whisky instead of drinking water. The whisky gave these rats, on the average, a 28% higher caloric intake than that of the rats that drank water. The average daily intake of diluted whisky was 3.7 cc.

*Results.* In eleven of the twelve pairs polyneuropathy first occurred in the rat drinking water (fig. 2). The twelfth pair was discarded because one of the rats (receiving whisky) died without observed polyneuropathy. There were no abnormal post mortem findings except emaciation. In the eleven pairs the average time between the withdrawal of thiamine chloride and the onset of polyneuropathy in the rats on water was 19 days; in the rats on whisky, 29 days (a 53% longer interval). The rat on water limited the diet intake in every pair.

During the 42-day preliminary period the rats on whisky in seven of the eleven pairs had a greater gain in body weight (average 11%) than the litter mates on water. In one pair the weight gain was equal. In three pairs the rats on water had a greater gain of weight (average 13%) than the litter mates on whisky. Following the withdrawal of thiamine a rapid decline in weight occurred in both the rats receiving water and those getting whisky.

EFFECT OF WHISKY WITH EQUAL FOOD INTAKE
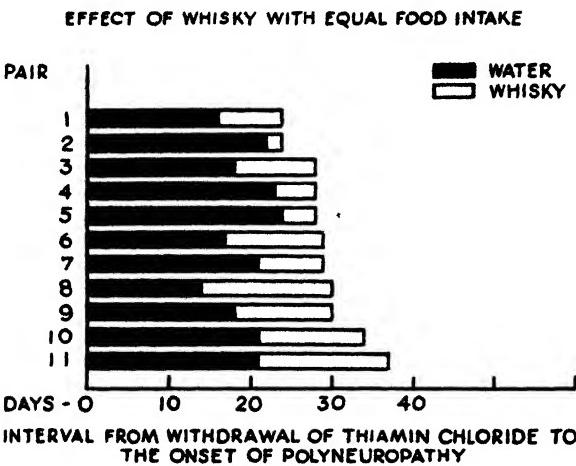


Figure 2

## Experiment III

### Effect of ethyl alcohol with equal food intake

This experiment is similar to experiment II. Throughout the experiment one rat in each pair was given alcohol ad libitum (approximately 20% by volume) instead of drinking water. The amount of alcohol taken was not recorded. Twenty-four pairs of litter mates were used. In each pair, the rats were fed an equal amount of diet no. 461 up to the time of development of polyneuropathy in one of the rats of the pair; the paired rat was then fed diet no. 461 ad libitum.

*Results.* In twenty-three of the twenty-four pairs, polyneuropathy first occurred in the rat on water (fig. 3). The

twenty-fourth pair was discarded because one of the rats (that got water) died without observed polyneuropathy and post mortem examination showed pneumonia. In the twenty-three pairs the average time between the withdrawal of thiamine chloride and the onset of polyneuropathy in the rats on water was 14 days; in the rats on alcohol, 22 days (a 57% longer interval). The rat on water limited the diet intake in every pair.
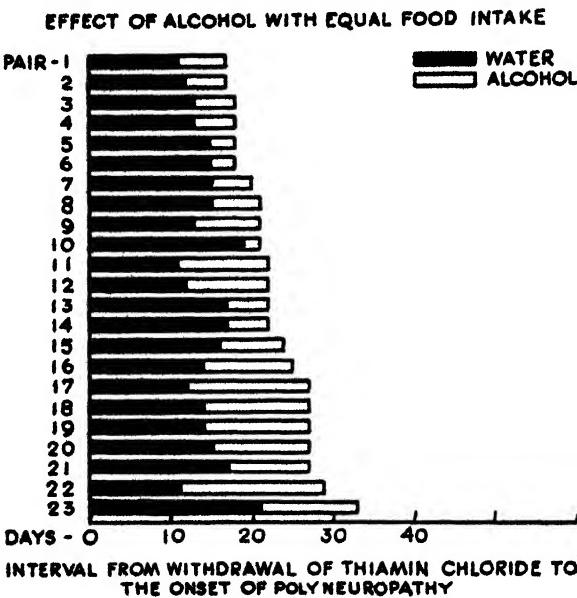


Figure 3

During the 42-day preliminary period the rats getting alcohol in nineteen of twenty-three pairs had a gain of weight that was greater (average 23%) than that of the litter mates receiving water. In one pair the gain was equal, and in three pairs the rats that drank water had a greater gain (average 13%) than the litter mates that received alcohol. After the withdrawal of the thiamine there was a rapid decline in body weight in both the rats getting alcohol and those drinking water.

## Experiment IV

### Effect of food restriction with ethyl alcohol

Twelve litters of three rats each were fed diet no. 461. In each litter, rat A was given drinking water and diet no. 461 ad libitum. Rat B was given alcohol and received the same amount of diet no. 461 as rat A until the thiamine was withdrawn, and was then allowed to eat ad libitum. Rat C was given alcohol and had the intake of diet no. 461 restricted to
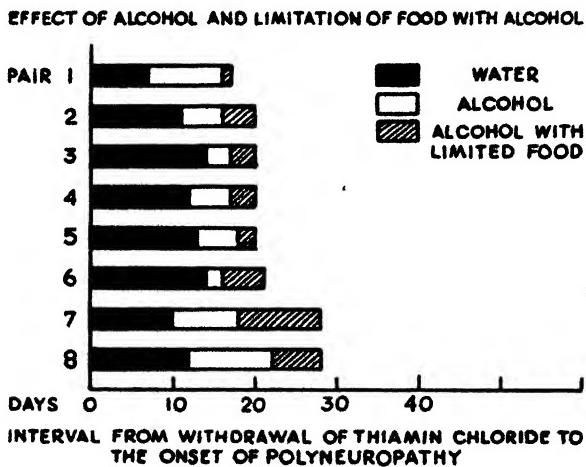


Figure 4

80% of the amount eaten by rat B. At the time of development of polyneuropathy in rat B, rat C was fed diet no. 461 ad libitum.

*Results.* In eight of the twelve litters polyneuropathy occurred first in rat A (on water), next in rat B (on alcohol) and last in rat C (on alcohol with a restricted diet intake) (fig. 4). In the remaining four litters polyneuropathy was not observed prior to death in at least one rat in each litter.

In the eight litters in which polyneuropathy was observed in all of the rats the average time from the withdrawal of thiamine chloride to the onset of the polyneuropathy in the rats on water (A) was 12 days; in the rats on alcohol (B)

18 days (a 50% longer interval) and in the rats on alcohol with a restricted food intake (C) 22 days (an 83% longer interval). The rat on water (A) limited the diet intake in every litter. The rats on water (A) had an abrupt decrease in the intake of food after the withdrawal of thiamine. The litter mates on alcohol (B) that had the same intake of diet no. 461 until the withdrawal of thiamine had a more gradual decrease in the amount of diet eaten daily. The rats on alcohol (B) consumed 26% more of diet no. 461 than the rats receiving water (A) from the time of withdrawal of the thiamine to the onset of polyneuropathy in the rat getting water (A). During this period the average daily diet consumption of the rats on water (A) was 1.9 gm., and of the rats on alcohol (B) 2.5 gm.

In the four litters in which polyneuropathy was not observed in at least one rat in each litter, one rat on alcohol (B) and three on alcohol with a restricted food intake (C) died without showing evidence of polyneuropathy. No pathological changes except emaciation were found at the post mortem examination. All four of the rats on water (A) died without showing evidence of polyneuropathy, and post mortem examination revealed a large accumulation of clear, colorless fluid in the thoracic and peritoneal cavities. The hearts appeared enlarged and both ventricles were filled with blood. The histological examination of the hearts of these four animals showed short or long areas of the auricular myocardium in which muscle fibers were absent or decreased in number with a consequent marked thinning of the wall in three animals. In such areas there was slight to moderate fibroblast proliferation, slight collagen deposition, and occasional to few lymphocytes. The auricle of the fourth heart was not present in the section examined. These lesions were not present in the ventricular myocardium.

## Experiment V

### Prevention and treatment of polyneuropathy by thiamine

Three litters of four rats each were fed diet no. 461. Two rats in each litter were given diluted whisky, and two were

given water. The four litter mates received an isocaloric intake based on the caloric value of the amount of diet no. 461 eaten plus the whisky. In each litter one rat on whisky and one rat on water were given 15 µg. of thiamine chloride daily by mouth added to the supplement of the other vitamins; one rat on whisky and one rat on water were on the thiamine deficient regime. (The rats on the thiamine deficient regime are the same animals reported under litters no. 6, 10 and 11 in experiment I). Twenty-five rats from experiments I–IV were given 200–400 µg. of thiamine chloride immediately after the first appearance of polyneuropathy.

*Results.* None of the rats on water or whisky that received 15 µg. of thiamine chloride daily showed any·signs of polyneuropathy. The litter mates on the thiamine deficient regime getting either water or whisky developed polyneuropathy as shown in table 1. (Several hundred rats receiving 20% ethyl

TABLE 1

*Days required for development of polyneuropathy.*

| LITTER | B₁ DEFICIENT REGIME AND RECEIVING | | B₁ DEFICIENT REGIME + 15 µG B₁ DAILY AND RECEIVING | |
|---|---|---|---|---|
| | Water | Whisky | Water | Whisky |
| 6 | 16 | 31 | No polyneuritis | No polyneuritis |
| 10 | 13 | 37 | No polyneuritis | No polyneuritis |
| 11 | 15 | 40 | No polyneuritis | No polyneuritis |

alcohol as a source of fluid and eating various diets containing adequate thiamine have been maintained in this laboratory for periods as long as 1 year without developing polyneuropathy.) In the twenty-five rats treated with thiamine chloride immediately after the onset of polyneuropathy, the symptoms disappeared within 24 hours whether the animal was on alcohol, whisky, or water.

### DISCUSSION

In paired feeding experiments (I–IV above) with a thiamine deficient diet, fifty-four rats on water without exception developed polyneuropathy before the paired litter mates on

alcohol or whisky. It is clear from the results of the above experiments that under these conditions alcohol and whisky caused a delay in the onset of the polyneuropathy. No definite explanation of the mechanism of this delay is evident. It is obvious that the data do not support the assumption that the ingestion of alcohol increases the thiamine requirement.

The results of the preventive and therapeutic experiment (V) demonstrate that the polyneuropathy observed was related to the thiamine deficiency and could be prevented or alleviated by the administration of thiamine regardless of the presence or absence of alcohol or whisky. It is interesting to note that the four rats on water in experiment IV that died without having polyneuropathy that was observed showed hydrothorax, ascites, and histopathological changes in the heart. Drury, Harris and Maudsley ('30) observed bradycardia, and Weiss, Haynes and Zoll ('38) have described electrocardiographic changes in thiamine deficient rats.

## CONCLUSION

Under the conditions of these experiments the ingestion of alcohol or whisky delays the onset of polyneuropathy in thiamine deficient rats.

## LITERATURE CITED

COWGILL, G. R. 1934 The Vitamin B Requirement of Man. New Haven. Yale University Press.

DRURY, A. N., L. J. HARRIS AND C. MAUDSLEY 1930 Vitamin B deficiency in the rat; bradycardia as a distinctive feature. Biochem. J., vol. 24, pp. 1632–1649.

EVANS, H. M., AND S. LEPKOVSKY 1929 The sparing action of fat on the antineuritic vitamin B. J. Biol. Chem., vol. 83, pp. 269–287.

———— 1934 The sparing action of fat on vitamin B; effectiveness of various natural fats in sparing vitamin B. J. Biol. Chem., vol. 107, pp. 439–442.

———— 1935 Sparing action of fat on vitamin B. VIII. On the loss of vitamin B from the rat's tissues. J. Biol. Chem., vol. 108, pp. 439–455.

JOLLIFFE, N., AND C. N. COLBERT 1936 The etiology of the polyneuritis in the alcohol addict. J. Am. Med. Assoc., vol. 107, pp. 642–647.

JOLLIFFE, N., C. COLBERT AND P. M. JOFFE 1936 Observations on the etiologic relationship of vitamin B(B₁) to the polyneuritis in the alcohol addict. Am. J. Med. Sc., vol. 191, pp. 515–526.

KEMMERER, A. R., AND H. STEENBOCK 1933 A study of the sparing action of fats on vitamin B content of animal tissues. J. Biol. Chem., vol. 103, pp. 353–362.

McHENRY, E. W. 1937 Effect of choline on vitamin B₁ sparing action of fats. Biochem. J., vol. 31, pp. 1616–1621.

SALMON, W. D., AND J. G. GOODMAN 1937 Alleviation of vitamin B deficiency in the rat by certain natural fats and synthetic esters. J. Nutrition, vol. 13, pp. 477–500.

WEISS, S., F. W. HAYNES AND P. M. ZOLL 1938 Electrocardiographic manifestations and cardiac effect of drugs in vitamin B₁ deficiency in rats. Am. Heart J., vol. 15, pp. 206–220.

# THE THIAMINE AND RIBOFLAVIN CONTENTS OF CITRUS FRUITS [1]

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Owing to the paucity of data concerning the thiamine and riboflavin contents of citrus fruit, the work reported herein was undertaken.

## THIAMINE

The comprehensive review by Daniel and Munsell ('37) gives the following values (rat growth assays) for grapefruit and oranges — 20 and 60 Sherman-Chase units per 100 gm., respectively. By use of the rat bradycardia method, the value of 0.4 International Unit (equal to 1.2 µg.) per gram has been found for grapefruit, oranges and tangerines (Baker and Wright, '35). In a recent rat growth study, grapefruit and oranges (Florida) have been reported (Booher and Hartzler, '39) to contain 72 and 78 µg. per 100 gm., respectively. The value of 111 µg. per 100 gm. for oranges has been reported by Pyke ('39), the method of analysis involving fluorophotometry.

The authors of this paper have analyzed specimens of Florida grapefruit and oranges purchased in New York during the season of November, 1939, through May, 1940, and Florida tangerines purchased in December, January and February of the same season.

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[1] Read on September 11, 1941, before the Division of Agricultural and Food Chemistry at the 102nd meeting of the American Chemical Society at Atlantic City, N. J. Published by permission of the American Chemical Society.

In the case of each sample taken, a standard box (1.6 bushel capacity, grapefruit and oranges, and 0.8 bushel for tangerines) was purchased and one-fifth of the number of fruit in a box was taken for analysis. Thus one sample may have been derived from a number of different trees.

The juice was expressed by a pressure, not by a reaming method. This mixed juice was then analyzed for its thiamine content. The method of analysis was in principle that of Hennessy and Cerecedo ('39) with certain modifications as suggested by Dr. W. L. Sampson of the Merck Institute. In short, the thiamine was adsorbed on permutit, eluted with acidified potassium chloride solution and oxidized to thiochrome by means of alkaline ferricyanide solution. The thiochrome was extracted from the alkaline aqueous solution by isobutanol and the intensity of its fluorescence measured in a Pfaltz and Bauer fluorophotometer, with prescribed "blank" correction.

The expressed juice was, prior to analysis, stored in stoppered bottles at 2°–5°C. for 12–24 hours to effect a sedimentation of the pulp. The supernatant pulp-free, although cloudy, juice was taken for analysis. It was adjusted to pH = 4 to 4.5, takadiastase added, incubated from 1 to 1.5 hours at 37–38°C., heated to boiling and cooled to 80°C. before being passed through the permutit column. It was found that the enzyme treatment was essential and that it made no difference whether the supernatant pulp-free juice or juice containing pulp was incubated with the enzyme. Hence, owing to the greater convenience, the procedure as mentioned was adopted.

The results are given in table 1. The size designations are the number of individual fruits which just fill the standard Florida box — 1.6 bushel for grapefruit and oranges, and 0.8 bushel for tangerines. Based on the average juice volume of these particular specimens the thiamine chloride hydrochloride or vitamin $B_1$ expressed in micrograms per fruit of the size indicated is 80 per pineapple orange, 91 per Valencia orange, 85 per seeded grapefruit, 81 per seedless grapefruit and 45 per tangerine.

*Vitamin B$_1$ content of certain citrus fruits.*

| FRUIT | SIZE | NUMBER OF BOXES SAMPLED | TOTAL NUMBER OF FRUITS TAKEN | VITAMIN B$_1$ [1] MICROGRAMS PER 100 ML. JUICE | | | |
|---|---|---|---|---|---|---|---|
| | | | | Highest | Lowest | Mean | S. D.[2] |
| Oranges | | | | | | | |
| Pineapple | 176 | 11 [3] | 385 | 76 | 54 | 65 | 6.1 |
| Valencia | 176 | 11 | 385 | 81 | 58 | 70 | 6.7 |
| Grapefruit | | | | | | | |
| Seeded | 70 | 9 | 126 | 47 | 18 | 35 | 8.6 |
| Seedless | 70 | 10 | 140 | 43 | 18 | 32 | 7.5 |
| Tangerines | 150 | 5 | 150 | 78 | 59 | 69 | 6.4 |

[1] Calculated as thiamine chloride hydrochloride.

[2] Standard deviation from the mean.

[3] Including one box of Parson Brown oranges, analysis of which yielded 63 µg. of vitamin B$_1$ per 100 ml. juice.

## RIBOFLAVIN

The literature gives a wide divergence in values for the riboflavin content of citrus fruit based on assays of but a few specimens. In the Daniel and Munsell ('37) survey, the values given for grapefruit and oranges are 40 and 35 Sherman-Bourquin units per 100 gm., respectively. These figures are, in the opinion of the present authors, too high (assuming one Sherman-Bourquin unit to be equal to about 2.5 µg. of riboflavin). On the other hand, the low values of 6.9 and 8.9 µg. per 100 ml. of orange juice have been reported (Kuhn, Wagner-Jauregg and Kaltschmitt, '34). These values are undoubtedly much too low owing to the fact that they were obtained by the lumiflavin method in 1934.

Recently by rat growth assay (Lanford, Finkelstein and Sherman, '41), the values of 20.0 and 27.8 µg. per 100 gm. have been reported for grapefruit and oranges, respectively, and in India (Wilson and Roy, '38) the contents of 24 µg. (grapefruit) and 59 µg. (orange) per 100 gm. have been published.

In the present study, the riboflavin content of representative specimens of oranges and of grapefruit was measured fluoro-

metrically by a simple direct method described below. Each lot of citrus fruit was purchased in New York City on the day it arrived from Florida through the period December 16, 1940, to May 12, 1941. As in the case of the thiamine measurements, the juice of one-fifth of the total number of grapefruit or oranges in a box was expressed and this mixed juice was taken as a sample.

The mixed expressed juice was stored in darkness at 2°–5°C. for 12 to 24 hours in completely filled glass bottles and then filtered through paper. This storage preliminary to filtration results in a flocculation of colloidal matter and greatly facilitates the production of a clear filtrate. Clarity is essential, because even a slight haze greatly increases the degree of quenching of the fluorescence. The first 50–75 ml. of filtrate were discarded and the filtration continued in a refrigerator at 2°–5°C., and, of course, in absence of light.

The intensity of fluorescence was measured by use of a fluorophotometer,[2] the incident light passing through a combination of Corning filters nos. 038 and 511. Between the cuvette and photronic cell there was placed a Corning filter no. 351.

When the Klett instrument was used, the opposing photronic cell was incited by the fluorescence of a quinine sulfate solution (0.15 mg. quinine sulfate per liter of tenth normal sulfuric acid solution). The incident light was filtered through a Corning no. 584 filter and the combination of filters nos. 038 and 428 was placed between the quinine solution and photronic cell.

The fluorescence of the following solutions was measured: (A) 25 ml. of filtered juice diluted to 50 ml. with distilled water; (B) 25 ml. of filtered juice diluted to 50 ml. with a standard riboflavin solution producing a riboflavin increment of 0.05 μg. per milliliter; (C) same as B except that riboflavin increment was 0.075 μg. per milliliter; (D) any two of A, B and C treated with hydrosulfite for "blank" correction.

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[2] Both the Pfaltz and Bauer and the Klett instruments were used.

The hydrosulfite solution (Sullivan and Norris, '39) was prepared by dissolving 5 gm. of sodium hydrosulfite in 100 ml. of an ice-cold sodium bicarbonate solution (2 gm. $NaHCO_3$ per 100 ml.). This solution was added to A, B or C in the proportion of 3 ml. of the former to 25 ml. of the latter.

A typical calculation may be helpful: The photometer scale readings of these solutions in one experiment were — A $= 121$, B $= 179$, C $= 210$ and D $= 34$. Hence, the readings corrected for the "blank" were — A $= 87$, B $= 145$, C $= 176$ and

$$\frac{0.05}{145-87} \times 87 = 0.0750; \quad \frac{0.075}{176-87} \times 87 = 0.0733$$

Thus the indicated riboflavin content of A is 0.074 µg. per milliliter and that of the filtered juice is 0.15 µg. per milliliter.

This method renders unnecessary the preparation of a standard of reference curve of photometer response plotted against riboflavin concentration unless one desires to know the extent to which the intensity of fluorescence of riboflavin is quenched by substances in the solution analyzed. In the example cited the degree of quenching was thus found to be 35%. Obviously, either solution B or C is sufficient for an analysis. The authors preferred, however, to run both B and C as "checks" at two levels of added riboflavin. All analytical operations were performed in a dimly lighted room.

The results by this method were compared with those obtained by use of the Snell and Strong ('39) microbiological method in three instances.

Specimens of filtered grapefruit juice (our specimen no. 634) and of filtered orange juice (our specimens no. 639 and 642) yielded the results of 14, 18 and 15 µg. of riboflavin per 100 ml., respectively, by the method described in this paper and 14, 18 and 14 µg., respectively, by the Snell and Strong method.[3]

Higher results were obtained, however, when the microbiological method was applied to unfiltered juice. The values of 22 and 18 µg. per 100 ml. were obtained for unfiltered specimens 639 and 642, respectively. Since these higher results (if

[3] The microbiological assays were kindly performed by Mr. Carol E. Weill.

valid) indicated that riboflavin might be either bound to constituents of the cellular structure of the pulp or adsorbed by the filter paper, further experiments were performed. It was found that the prescribed conditions of filtration were not at fault.

Several extractive procedures were applied to the unfiltered juice. A specimen of orange juice was heated by immersion in boiling water for 1.5 hours under a reflux condenser. It was cooled and filtered. The filtrate was darker in color than the original juice and gave a lower value for riboflavin. The fluorescence intensity after addition of hydrosulfite had increased also.

Since Hodson and Norris ('39) recommend extraction of solid samples with 0.25 N sulfuric acid, a specimen of unfiltered orange juice was rendered 0.25 N with respect to sulfuric acid and heated in boiling water for 25 minutes. Upon cooling, it was brought to pH = 5.53 by addition of saturated trisodium phosphate solution, diluted to a definite volume with distilled water and filtered. The filtrate gave within the error of the method the same riboflavin result as the untreated juice.

Acetone has been recommended as an extractant for milk by Hand ('39) who states also that at an acetone concentration of 66%, riboflavin combined with protein is disassociated therefrom. It was therefore considered of interest to try the effect of acetone upon citrus juice. Orange juice (unfiltered) and acetone were mixed at room temperature in the proportions of one volume of the former to two volumes of the latter. The filtrate was yellowish brown in color and the indicated riboflavin content of the juice was much lower than that of the untreated, filtered juice. Another result of the acetone treatment was the high fluorescence quenching power of the acetone soluble matter—the fluorescence intensity was quenched 85%; hence, no quantitative calculations would be acceptable.

Refluxing equal volumes of methanol and unfiltered orange juice for 25 minutes followed by cooling and filtering yielded a very satisfactory filtrate in which the fluorescence was quenched to the extent of only 26%. The riboflavin content

found by this method was the same as by direct measurement of untreated filtered juice.

The riboflavin content of certain citrus fruits as determined by the simplified method described herein is given in table 2.

TABLE 2

*Riboflavin content of certain citrus fruits.*

| FRUIT | SIZE | NUMBER OF BOXES SAMPLED | TOTAL NUMBER OF FRUITS TAKEN | RIBOFLAVIN MICROGRAMS PER 100 ML. JUICE | | | |
|---|---|---|---|---|---|---|---|
| | | | | Highest | Lowest | Mean | S. D.[1] |
| Oranges | | | | | | | |
| Pineapple | 176 | 7 | 245 | 17 | 13 | 16 | 1.3 |
| Valencia | 176 | 7 | 245 | 18 | 15 | 15 | 1.1 |
| Grapefruit | | | | | | | |
| Seeded | 70 | 8 | 112 | 14 | 10 | 12 | 1.4 |
| Seedless | 70 | 8 | 112 | 12 | 9 | 11 | 1.1 |

[1] Standard deviation from the mean.

Based on the average juice volume of these particular specimens, the riboflavin per fruit (of the size designated) is 20 μg. per orange, and 27 μg. per grapefruit.

### LITERATURE CITED

BAKER, A. Z., AND M. D. WRIGHT 1935 The vitamin B$_1$ content of foods. Biochem. J., vol. 29, p. 1802.

BOOHER, L. E., AND E. R. HARTZLER 1939 The vitamin B$_1$ content of foods in terms of crystalline thiamin. U. S. Dept. Agric., Tech. Bull. No. 707.

DANIEL, E. P., AND H. E. MUNSELL 1937 Vitamin content of foods; a summary of the chemistry of vitamins, units of measurement, quantitative aspects in human nutrition and occurrence in foods. U. S. Dept. Agric., Misc. Pub. No. 275.

HAND, D. B. 1939 Determination of riboflavin in milk by photoelectric fluorescence measurements. Ind. Eng. Chem., Anal. Ed., vol. 11, p. 306.

HENNESSY, D. J., AND L. R. CERECEDO 1939 The determination of free and phosphorylated thiamin by a modified thiochrome assay. J. Am. Chem. Soc., vol. 61, p. 179.

HODSON, A. Z., AND L. C. NORRIS 1939 A fluorometric method for determining the riboflavin content of foodstuffs. J. Biol. Chem., vol. 131, p. 621.

KUHN, R., T. WAGNER-JAUREGG AND H. KALTSCHMITT  1934  Über die Verbreitung der Flavine im Pflanzenreich. Ber., vol. 67, p. 1452.

LANFORD, C. S., B. FINKELSTEIN AND H. C. SHERMAN  1941  Riboflavin contents of some typical fruits. J. Nutrition, vol. 21, p. 175.

PYKE, M.  1939  The distribution of vitamin-$B_1$ in foods as determined by chemical analysis. J. Soc. Chem. Ind., vol. 58, p. 338.

SNELL, E. E., AND F. M. STRONG  1939  A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed., vol. 11, p. 346.

SULLIVAN, R. A., AND L. C. NORRIS  1939  Determining riboflavin in dried milk products. Ind. Eng. Chem., Anal. Ed., vol. 11, p. 535.

WILSON, H. E. C., AND G. K. ROY  1938  Indian J. Med. Res., vol. 25, p. 879, through M. A. B. Fixsen and M. H. Roscoe. 1940. Tables of the vitamin content of human and animal foods. Nutrition Abstracts and Rev., vol. 9, p. 795.

# STORAGE OF PYRIDOXINE IN THE RAT [1]

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Although it is generally assumed that animals have a limited capacity for storing the various factors of the vitamin B complex, little evidence has been presented to demonstrate this fact. We have observed that weanling rats on pyridoxine-free rations require a much longer period to develop dermatitis when they are allowed to continue feeding on the stock diet and thus increase their weight over that of controls. This suggested a capacity for storing pyridoxine. Such capacity was demonstrated when graded doses of this factor were administered over an initial 10-day period to rats maintained on the pyridoxine-free diet. The more vitamin the rats obtained during this period, the longer were they able to resist the onset of dermatitis. It is the object of this paper to present data which substantiate these findings.

## EXPERIMENTAL

Rats of the Wistar strain, reared in our laboratory, were employed. The basal diet (diet A) had the following percentage composition: casein, vitamin free [2] 30, sucrose 55, hydrogenated cottonseed oil [3] 5, cod liver oil 3, Osborne and Mendel salt mixture 7. Supplements of 10 µg. of thiamine chloride, 20 µg. of riboflavin, and 0.3 cc. of a fuller's earth filtrate of pig liver extract [4] equivalent to 3 gm. of fresh liver were fed daily.

Normally, rats weighing about 40 gm. at weaning, when placed immediately on our pyridoxine-free ration, develop

[1] Presented at the meeting of the American Chemical Society at Detroit, September, 1940.
[2] Labco.　[3] Crisco.　[4] Prepared by the method of Lepkovsky et al. ('36).

dermatitis in about 1 month. However, one group of animals, allowed to continue on the stock diet [5] for 10 days after weaning before they were given the deficient diet, averaged 51 gm. of body weight and did not develop dermatitis until after $2\frac{1}{2}$ months. This observation led us to select litters of varying ages and body weights and to compare their growth and the time of appearance of dermatitis on the pyridoxine-free diet. The results obtained are given in table 1. They show that with increasing initial body weight the incidence of dermatitis was correspondingly delayed.

TABLE 1

*Effect of initial body weight on growth and the time of appearance of dermatitis.*

| NO. OF RATS | AVERAGE INITIAL BODY WEIGHT | AVERAGE TIME OF APPEARANCE OF DERMATITIS | AVERAGE GAIN IN WEIGHT OVER 40 DAYS |
|---|---|---|---|
| | gm. | days | gm./day |
| 6 | 51 | 77 | 0.55 |
| 8 | 41 | 40 | 0.43 |
| 5 | 36 | 35 | 0.33 |
| 7 | 21 | 28 | 0.48 |

TABLE 2

*The growth and time of appearance of dermatitis in young rats whose mothers were depleted during the lactation period.*

| NO. OF RATS | PORTION OF THE LACTATION PERIOD USED | INITIAL BODY WEIGHT | AVERAGE TIME OF APPEARANCE OF DERMATITIS | AVERAGE GROWTH OVER 30 DAYS |
|---|---|---|---|---|
| | | gm. | days | gm./day |
| 40 | None | 38 | 33 | 0.33 |
| 4 | $\frac{1}{4}$ | 35 | 24 | 0.27 |
| 16 | $\frac{3}{4}$ | 25 | 19 | 0.17 |

In order to limit storage of pyridoxine prior to weaning, three nursing rats and their progeny were given the pyridoxine-free diet during the latter part of the lactation period. As indicated in table 2, these animals were of lower body weight and developed dermatitis at an earlier age than did normally reared young.

In the preceding experiments it has been shown that the higher the initial body weight of the rats when the animals are placed on the pyridoxine-free diet, the longer is the time

[5] Purina Dog Chow.

required for dermatitis to appear. It must be assumed that this increased body weight represents a proportional increase in the amount of pyridoxine accumulated in the tissues, and that the animal uses this factor sparingly. However, since there may be other factors that influence the appearance of dermatitis, this assumption is not entirely justified. Analysis of the body tissues for pyridoxine after increasing periods of time on the deficient ration would have furnished evidence of storage, but unfortunately the methods available at present for the estimation of pyridoxine are not sufficiently dependable. We, therefore, decided to select animals of the same body weight, give them graded doses of pyridoxine during a short initial period, and then observe whether the time of appearance of dermatitis could be related to the amount of the vitamin that they received.

A total of thirty-six rats were given diet A at weaning, and, after a 5, 10, or 15 day depletion period, were divided into four groups and given the pyridoxine-free diet (diet A plus the supplements). These four groups then received 0, 5, 10, and 15 µg., respectively, of pyridoxine over a 10-day period, after which this supplement was discontinued. The animals showed an increased growth rate and a delay in the appearance of dermatitis corresponding to the amount of pyridoxine initially fed (table 3).

This experiment was repeated with a group of twenty rats receiving diet A supplemented with 2 mg. of thiamine chloride, 4 mg. of riboflavin, and 20 mg. of d-calcium pantothenate [6] per kilo of diet. The animals were divided into four groups and given 0, 5, 10, and 15 µg., respectively, of pyridoxine daily for 10 days, after which time they were allowed to continue on the basal diet until dermatitis developed. The results, as shown in table 3, were the same as those obtained in the preceding experiment.

### SUMMARY

The storage capacity for pyridoxine has been studied on young rats maintained on a vitamin B complex free diet

TABLE 3

*The relation of growth and the time of appearance of dermatitis to the amount of pyridoxine ingested over an initial 10-day period.*

| NO OF RATS | DEPLETION PERIOD | DAILY DOSE OF PYRIDOXINE | TIME OF APPEARANCE OF DERMATITIS | AVERAGE GAIN IN WEIGHT OVER 40 DAYS |
|---|---|---|---|---|
|  | *days* | *μg* | *days* | *gm /day* |
| 4 | 5 | 15 | 59 | 1.3 |
| 4 | 5 | 5 | 43 | 0.9 |
| 4 | 5 | 0 | 28 | 0.5 |
| 4 | 10 | 15 | 58 | 0.9 |
| 4 | 10 | 5 | 45 | 0.55 |
| 4 | 10 | 0 | 30 | 0.2 |
| 4 | 15 | 15 | 70 | 0.9 |
| 4 | 15 | 10 | 60 | 0.8 |
| 4 | 15 | 0 | 35 | 0.47 |
| 5 [1] | 10 | 15 | 68 | 1.9 |
| 5 [1] | 10 | 10 | 56 | 1.75 |
| 5 [1] | 10 | 5 | 45 | 1.25 |
| 5 [1] | 10 | 0 | 29 | 0.95 |

[1] These animals were given pantothenic acid in place of liver filtrate.

supplemented with thiamine, riboflavin, and liver filtrate (or pantothenic acid).

If the rats are weanlings, they develop dermatitis in about 1 month; if they are allowed to subsist on the stock diet before being fed the pyridoxine-free ration, dermatitis does not develop until after $2\frac{1}{2}$ months. If the mother is given the $B_6$-free diet during the latter part of the lactation period, the young grow more slowly and symptoms of $B_6$ deficiency appear earlier than otherwise. Depleted animals given stated amounts of pyridoxine over a 10-day period show a progressive increase in rate of growth and corresponding delay in onset of dermatitis correlated with the amount of the vitamin given.

These data suggest that the irregularity in the occurrence of dermatitis in rats deprived of pyridoxine (reported by several workers) may be ascribed, at least in part, to differences in the reserves of the vitamin present in the young animals at the beginning of the experiment.

### LITERATURE CITED

LEPKOVSKY, S., T. H. JUKES AND M. E. KRAUSE 1936 The multiple nature of the third factor of the vitamin B complex. J. Biol. Chem., vol. 115, p. 557.

# THE MINIMUM VITAMIN A REQUIREMENT
# OF THE FOX

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A few experimental studies have been made of the qualitative need of the various vitamins by foxes. This report is the first one setting forth the quantitative need of a vitamin by the fox. It is now known that foxes need vitamin D (Smith and Barnes, '42), anti-grey hair vitamin (Lunde and Kringstad, '39; Morgan and Simms, '40), vitamin $B_1$ (Green, Carlson and Evans, '41; Carlstrom and Jonsson, '38; Ender and Helgebostad, '39; Hodson and Smith, unpublished data), and nicotinic acid (Hodson and Loosli, '42), and that they do not need vitamin C in their diets (Mathiesen, '39).

Coombes, Ott and Wisnicky ('40) fed ranch fox pups a diet low in vitamin A over a period of 210 days but they observed nothing distinctly abnormal. The diet while low, contained significant amounts of vitamin A. Holmes, Tripp, Ashbrook and Kellogg ('41) studied the liver storage of vitamin A in ranch-raised silver foxes and trapped wild foxes. The former had a range of 1.3 to 10.9 lovibond blue units of vitamin A per gram of liver as compared to an average of 326 blue units in the latter. A preliminary account of vitamin A deficiency in fox pups was made by Smith ('41). The investigations herein reported extend these observations and in addition include the results of studies of the minimum vitamin A requirement of growing pups.

## MATERIALS AND METHODS

This study has extended over a period of 3 years and has involved thirty-four silver fox pups. The first two experiments were concerned with a determination of the qualitative need for vitamin A, which included a detailed study of the deficiency symptoms. In the third year sixteen fox pups were used to determine the minimum vitamin A requirement necessary to prevent the deficiency symptoms in growing pups. The general plan of all of these studies has been to place nursing dams on a diet lacking rich sources of vitamin A so that a large supply of vitamin A would not be built up in the pups. The young were weaned at 6 weeks of age and placed on the experiments.

The basal, vitamin A deficient diet used in these studies had the following percentage composition: meat scrap 30, skim milk powder 7, oatmeal 30, dried yeast [2] 5, cottonseed oil [3] 5, steamed bone meal 2, salt mixture 1 and water 20. To this diet were added irradiated yeast and ascorbic acid, the latter being purely precautionary. The meat scrap (65% protein) and skim milk powder were spread in thin (2 inches) layers and heated at approximately 70° C. for 4 days to insure destruction of traces of vitamin A. The salt mixture consisted of 90% sodium chloride and 10% ferrous sulphate. This diet when fed to 3-week-old rats produced a delayed but characteristic vitamin A deficiency, whereas the control rats supplemented with cod liver oil grew and remained healthy.

The experimental pups were placed in 4-foot by 6-foot wire-floored pens and fed the basal diet ad libitum. Vitamin A supplements when given were administered by mouth as is discussed later. Fresh water was available at all times. Tetrachlorethylene was given as a vermifuge and the pups were treated for ear mites as often as necessary by swabbing out the ears with a mixture of tincture of iodine, ether and glycerol in equal parts. The foxes were weighed at weekly

[2] Product of the Northwestern Yeast Co.
[3] Wesson.

intervals for the first 3 months and thereafter at biweekly intervals. When the foxes died or were killed the livers were tested for vitamin A, using the Carr-Price reaction ('26). Blood for vitamin A determinations was obtained by cardiac puncture. The vitamin A determinations were made by the technic of Kimble ('39), except that a photo-electric colorimeter which had previously been standardized with crystalline vitamin A was used. With the exception of the nervous tissue, the various tissues studied were fixed in Bouin's fluid and stained with hematoxylin and eosin. The nervous tissue was fixed in 10% formaldehyde and stained by a modified Marchi technic (Swank and Davenport, '35). In a few cases the brain was perfused with a potassium bichromate-magnesium sulphate solution before removal. The nervous tissue was imbedded in celloidin and then sectioned.

## SYMPTOMS OF VITAMIN A DEFICIENCY

All of the eighteen pups which received the basal vitamin A deficient diet developed a series of nervous derangements which usually began with a trembling of the head. This was followed shortly by what we have termed head "cocking," an action resembling that of a chicken observing a hawk or an airplane flying overhead. The sense of balance was definitely disturbed, for the affected pups, in viewing an object behind them, jerked their heads over their shoulders instead of turning around in the normal fashion. Often in doing this they lost their balance completely and rolled over. In addition, ten of the pups ran rapidly in circles especially when they were excited. This occurred frequently and often lasted for 10 to 15 minutes at a time. The severity of the symptoms varied but appeared to be worst on hot days. The time of onset of the symptoms varied from 1 to 5 months after the start of the experiments, but most of the pups showed the first symptoms within 2 months.

A more delayed symptom (2 to 4 months) which occurred in four of the foxes was that of coma. These pups would appear to be losing their balance, would waver and drop to

the floor and become completely unconscious. Occasionally there was some thrashing of the legs. These seizures lasted usually for 5 to 15 minutes, after which the pups would shakily arise and assume normal attitudes. These comas sometimes occurred spontaneously but usually they followed periods of excitement caused by handling or pounding on the pens.

Six pups were observed for longer periods of time, and of these, three developed typical xerophthalmia (fig. 2) at the eighteenth, twenty-fifth and twenty-seventh week following the start of the experiment. Unilateral at first, this soon affected both eyes in all three cases. These foxes were killed soon after the appearance of xerophthalmia but not before ulceration and rupture of the eyeballs had occurred. Papillary edema was definitely observed in two foxes in the more chronic stages of the deficiency. One female fox came into estrus and was bred. She conceived but aborted a litter of five well-formed fetuses at the forty-third day of pregnancy (normal gestation 52 days).

Microscopic examination of various epithelial tissues did not show any metaplasia as noted in other animals (Wolbach and Howe, '25) until approximately at the time of appearance of xerophthalmia. At this time stratification and keratinization of the epithelium of the cornea, trachea (fig. 3), bronchi, kidney pelvis, urinary bladder and vagina (fig. 4) were observed. Vaginal smears were taken twice weekly from a group of six female pups and in no case did cornified epithelial cells appear until the very late stages of the deficiency. In these animals the nervous symptoms preceded the vaginal cornification by several weeks. The epithelial changes thus appear considerably later than the nervous disorders which are the first clinical symptoms of vitamin A deficiency in the fox. Nerve fiber degeneration was observed in several parts of the nervous system which thus explains the clinical observations. This phase of the study is to be reported in detail elsewhere and only the more salient points will be mentioned here. There was widespread myelin degeneration in the spinal cord (fig. 5) and in the VIII cranial nerve (fig. 6) in which

both the cochlear and vestibular divisions were involved. Mellanby ('38) has made similar observations in vitamin A deficient dogs. The foxes must have been deaf though the clinical detection of this was difficult. This disturbance of the vestibular apparatus undoubtedly explains the upset sense of balance of the pups. Areas of the brain which have so far shown myelin degeneration are the basal ganglia, nuclei of the VIII nerve and many fiber tracts concerned with motor sense. Overgrowth of the cranial and vertebral bones as a contributing factor to the nerve degenerations as observed by Mellanby ('38, '41) in vitamin A deficient dogs was not investigated here.

Liver samples (15 to 35 gm.) of the foxes which received the unsupplemented basal diet in every case failed to show detectable amounts of vitamin A by the Carr-Price reaction, whereas those foxes which received certain levels of vitamin A supplements did show liver storage (table 1).

Attempts to cure the nervous deficiency symptoms have failed, even when 50,000 I. U. of vitamin A were given twice weekly over a period of 3 weeks. Other symptoms of the deficiency, such as loss of appetite and lethargy, were improved by these administrations. Two foxes which developed the nervous symptoms were subsequently placed on a complete diet fed to the stock foxes. Even after a period of 1 year these two foxes still showed nervous disturbances. These results are in keeping with the fact that nerve injuries of the type involved here are irreparable once established.

### THE MINIMUM REQUIREMENT

The minimum requirement is defined as the amount of vitamin A, calculated to a daily dose, that just prevents clinical symptoms of vitamin A deficiency. This amount has been found adequate for good growth and general well-being.

On June 2, 1941, sixteen fox pups used in this study were divided into six groups, five of which contained three each. These groups consisted of a negative control group which received the vitamin A deficient diet only; four groups which

received, respectively, 15, 25, 50 and 100 I. U. of vitamin A per kilogram of body weight per day, and a positive control group which, in addition to the basal diet received a cod liver oil concentrate (about 8,000 I. U. of vitamin A per pup per day). With the exception of the last group the vitamin A supplement was U. S. P. reference cod liver oil which was administered by mouth twice weekly. The dose was adjusted after each weekly weighing.

The results are summarized in table 1. The nervous symptoms characteristic of vitamin A deficiency appeared in all members of the negative control group and in two of the three foxes which received 15 I. U. of vitamin A per kilogram per day. All groups receiving more vitamin A than this failed

TABLE 1

*The results of the studies of the minimum vitamin A requirement of growing foxes.*

| GROUP | FOX | START OF DEFICIENCY SYMPTOMS | VITAMIN A IN THE | | REMARKS |
|---|---|---|---|---|---|
| | | | Liver | Blood | |
| | | *weeks* | *µg /100 gm.* | *µg./ml.* | |
| Negative | 46 | 13 | 0 | .. | Killed 12/15/41. |
| control | 40 | 11 | 0 | .. | Moribund 11/26. Killed. |
| | 52 | 11 | 0 | .. | Moribund 10/4. Killed. |
| 15 I. U.[1] | 57 | 25 | 0 | 0 | Killed 12/15/41. |
| | 53 | 11 | 0 | 0 | Killed 12/15/41. |
| | 49 | .. | 0 | 0 | Killed 12/15/41. |
| 25 I. U. | 50 | .. | 0 | Trace | Killed 12/15/41. |
| | 47 | .. | Trace | 0 | Killed 12/15/41. |
| | 54 | .. | 0 | .. | Died 7/25 from heat collapse. |
| 50 I. U. | 55 | .. | 70 | Trace | Killed 12/15/41. |
| | 41 | .. | 0 | 0.34 | Killed 12/15/41. |
| | 42 | .. | .. | 0.34 | Died 12/6. Cause unknown. |
| 100 I. U. | 48 | .. | 33 | 0.55 | Killed 12/15/41. |
| Positive | 51 | .. | 125 | 1.30 | Killed 12/15/41. |
| control | 56 | .. | 888 | 0.77 | Killed 12/15/41. |
| | 45 | .. | 825 | 0.35 | Killed 12/15/41. |

[1] International units of vitamin A per kilogram of body weight per day.

to show any indication of vitamin A deficiency. The minimum vitamin A requirement thus lies between 15 and 25 I. U. per kilogram of body weight per day.

The vitamin A content of the liver and blood plasma was variable, which is to be expected with such small numbers. The trends, however, are evident. No appreciable liver storage of vitamin A occurred until 50 to 100 I. U. of vitamin A per kilogram per day was administered. The positive control pups which received about 8,000 I. U. per pup per day had large liver stores of vitamin A. Detectable amounts of vitamin A were found in the liver and blood in one fox of the group which received 25 I. U. per kilogram per day, and in increasing amounts in the groups which received higher levels. The three positive control foxes averaged 0.81 µg. of vitamin A per milliliter of blood plasma.

Foxes 40 and 52 were killed in a moribund condition so that the brains could be removed free of postmortem changes. Fox 54 died after being handled on a very hot day in July; the symptoms indicated heat collapse, a condition to which foxes are apparently very sensitive. Fox 42 died in the late stages of the experiment from an unknown cause. This fox gave no clinical indications of vitamin A deficiency and the blood contained significant amounts of vitamin A. Unfortunately the liver sample was lost.

The curve of average growth of the positive control foxes is compared to that of the negative control foxes in figure 1. It will be noted that the growth rate declined in the late stages of vitamin A deficiency, associated with a decreased appetite and listlessness. It was surprising to note, however, that growth was so good in the negative control foxes, the decline not occurring until some weeks after the onset of the nervous symptoms. This was also noted in the experiments of the previous years. The growth of the foxes receiving 25 and 50 I. U. was as good as that of the positive control group.

Of course the fur ranchers are interested in the quality of the furs produced by foxes. The small numbers of animals studied here do not permit a critical comparison of the fur

of vitamin deficient and normal animals. The furs of the six foxes in the negative control group and the 15 I. U. group were not distinctly different, however, from those of the remaining foxes in this study. So far as can be seen from this study vitamin A deficiency has no specific effect on fur quality.
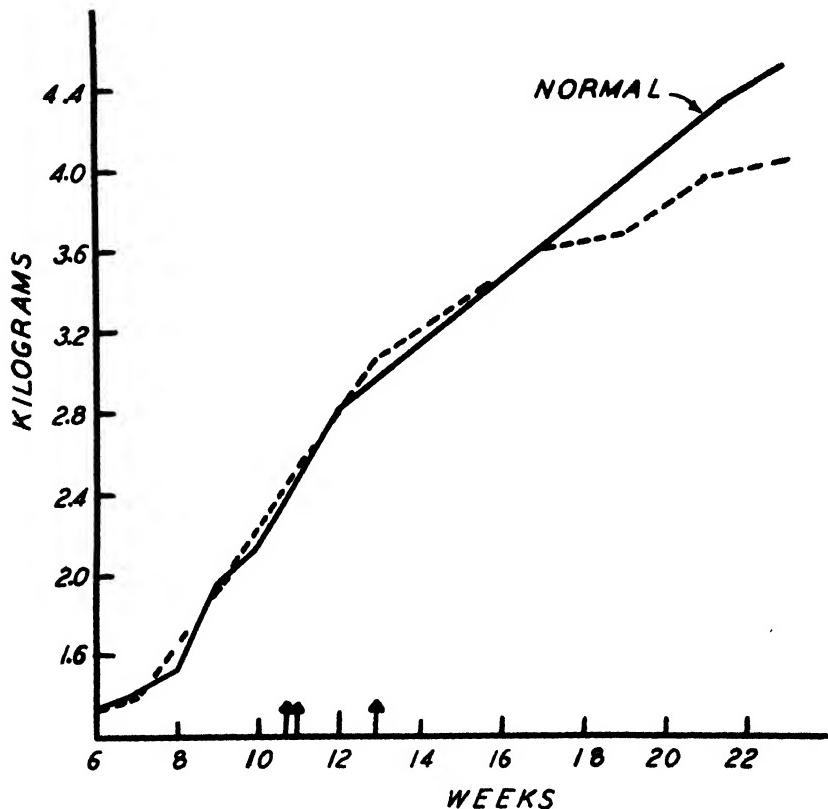


Fig. 1 The curves of average growth of the positive control and negative control foxes. The pups were started on experiment at 6 weeks of age. The arrows indicate the onset of the nervous deficiency symptoms in the negative control foxes.

## DISCUSSION

The results of these studies agree well with the minimum vitamin A requirement of cattle, sheep, swine, horses and rats (Guilbert, Howell and Hart, '40). They also lend support to the statement of the foregoing authors that the vitamin A

requirement is more nearly related to the body weight of mammals than to the energy requirements. These authors showed that this observation held for members of the ungulates and rodents whereas this study extends it to a carnivore.

With some slight modifications, the symptoms displayed by vitamin A-deficient foxes have their parallel in symptoms exhibited by other vitamin A deficient animals (Hart and Guilbert, '37; Hart, '40), that is, nervous disturbances, xerophthalmia, abortions, and epithelial metaplasia. Methods for testing night blindness in foxes unfortunately have not been successful. The nervous running in circles was noted also in vitamin A deficient dogs (Frohring, '35).

That a cure of the nervous disturbances did not follow the administration of vitamin A is not surprising for, as Hart and Guilbert stated, "Lesions in nerves once demonstrated are usually permanent and probably often progressive." Zimmerman and Cowgill ('36) were unable to effect a cure of demyelinized nerve fibers caused by vitamin A deficiency in the rat.

Of the farm animals, the vitamin A-deficient pig exhibits a syndrome more nearly like that of vitamin A-deficient foxes. Hughes, Lienhardt and Aubel ('29) stated that, "With swine, we have found the incoordination to be the outstanding symptom of avitaminosis A, while the eye lesions, which are so prominent in the rat, are of little importance." In the fox the neurological symptoms were the earliest and most evident signs of a deficiency of vitamin A.

It should be emphasized that the requirement here defined is a minimum requirement. The requirement for other bodily functions, notably reproduction, is undoubtedly higher. For practical purposes several times the minimum requirement should be fed.

## SUMMARY

Experimental vitamin A deficiency in the fox is characterized by nervous disturbances — trembling and "cocking" of the head, periods of whirling and in some cases coma, xeroph-

thalmia, widespread epithelial metaplasia, demyelinization of many nerve fibers and abortions. The earliest signs of a deficiency of vitamin A are the nervous symptoms. The growth of deficient animals while good at first, declined in the late stages. No specific effect of avitaminosis A was noted on the quality of the fur.

The minimum vitamin A requirement necessary to prevent the occurrence of the nervous symptoms in growing pups lies between 15 and 25 I. U. per kilogram of body weight per day. Storage of vitamin A did not occur in the liver until 50 to 100 I. U. of vitamin A per kilogram per day was fed.

LITERATURE CITED

CARLSTROM, B., AND G. JONSSON 1938 Ataxia, konvulsioner och acetonemi i samband med B- avitaminos. Skand. Veterinar-Tidskrift, vol. 28, p. 144.

CARR, F. H., AND E. A. PRICE 1926 Colour reactions attributed to vitamin A. Biochem. J., vol. 20, p. 497.

COOMBES, A. I., G. L. OTT AND W. WISNICKY 1940 Vitamin A studies with foxes. North Amer. Vet., vol. 21, p. 601.

ENDER, F., AND A. HELGEBOSTAD 1939 Experimental beri-beri in silver foxes. Skand. Vet., vol. 29, p. 1232.

FROHRING, W. O. 1935 Vitamin A requirement of growing puppies. Proc. Soc. Exp. Biol. Med., vol. 33, p. 280.

GREEN, R. G., W. E. CARLSON AND C. A. EVANS 1941 A deficiency disease of foxes produced by feeding fish. J. Nutrition, vol. 21, p. 243.

GUILBERT, H. R., C. E. HOWELL AND G. H. HART 1940 Minimum vitamin A and carotene requirements of mammalian species. J. Nutrition, vol. 19, p. 91.

HART, G. H., AND H. R. GUILBERT 1937 Symptomology of vitamin A deficiency in domestic animals. J. Amer. Vet. Med., vol. 91, p. 193.

HART, G. H. 1940 Vitamin A deficiency and requirements of farm mammals. Nutrition Absts. Revs., vol. 10, p. 261.

HODSON, A. Z., AND J. K. LOOSLI 1942 Experimental nicotinic acid deficiency in the adult silver fox. In press.

HOLMES, A. D., F. TRIPP, F. G. ASHBROOK AND C. E. KELLOGG 1941 A study of vitamin A in the nutrition of foxes. Wildlife Research Bulletin, No. 3.

HUGHES, J. S., H. F. LIENHARDT AND C. E. AUBEL 1929 Nerve degeneration resulting from avitaminosis A. J. Nutrition, vol. 2, p. 183.

KIMBLE, M. S. 1939 The photocolorimetric determination of vitamin A and carotene in human plasma. J. Lab. Clin. Med., vol. 24, p. 1055.

LUNDE, G., AND H. KRINGSTAD 1939 Bedarf des Fuches au dem antigrauhaarfaktor, vitamin Bx. Naturwissenschaften, vol. 27, p. 755.

MATHIESEN, E. 1939 Do silver foxes need vitamin C? Vara Palsdjur, December.

MELLANBY, E.   1938   The experimental production of deafness in young animals by diet.  J. Physiol., vol. 94, p. 380.

———— ———— 1941  Skeletal changes affecting the nervous system produced in young dogs by diets deficient in vitamin A.  J. Physiol., vol. 99, p. 467.

MORGAN, A. F., AND H. D. SIMMS   1940   Anti-grey hair vitamin deficiency in the silver fox.  J. Nutrition, vol. 20, p. 627.

SMITH, S. E., AND L. L. BARNES   1942   Experimental rickets in silver foxes and minks.  In press.

SMITH, S. E.   1941   Vitamin A deficiency and how it affects silver foxes.  Fur and Market J., vol. 19, No. 6, p. 11.

SWANK, R. L., AND H. A. DAVENPORT   1935   Chlorate-osmic-formalin method for staining degenerating myelin.  Stain Tech., vol. 10, p. 87.

WOLBACH, S. B., AND P. R. HOWE   1925   Tissue changes following deprivation of fat soluble A vitamin.  J. Exper. Med., vol. 42, p. 753.

ZIMMERMAN, H. M., AND G. R. COWGILL   1936   Lesions in the nervous system in vitamin deficiency.  IV. The effect of carotene in the treatment of the nervous disorder in rats fed a diet low in vitamin A.  J. Nutrition, vol. 11, p. 411.

2   Xerophthalmia in a vitamin A deficient fox.

3   This shows a stratified, squamous epithelium lining the bronchus of a vitamin A deficient fox. The normal epithelium is a pseudostratified, ciliated epithelium.

4   This shows the highly stratified, squamous epithelium, much of which is desquamating, in a vitamin A deficient female fox. This section was taken in the fall when vixens are in anestrum. The normal vagina at this time has an epithelial layer consisting of one to three layers of cuboidal cells.

5   Cross-section of the spinal cord of a vitamin A deficient fox showing widespread demyelinization of the nerve fibers. Marchi stain.

6   Cross-section of the medulla oblongata of a vitamin A deficient fox. The section is through the trapezoid body and the demyelinization of the fibers of the VIII nerve is evident. Marchi stain.

PLATE 1

VITAMIN A REQUIREMENT OF FOXES
SEDGWICK F. SMITH
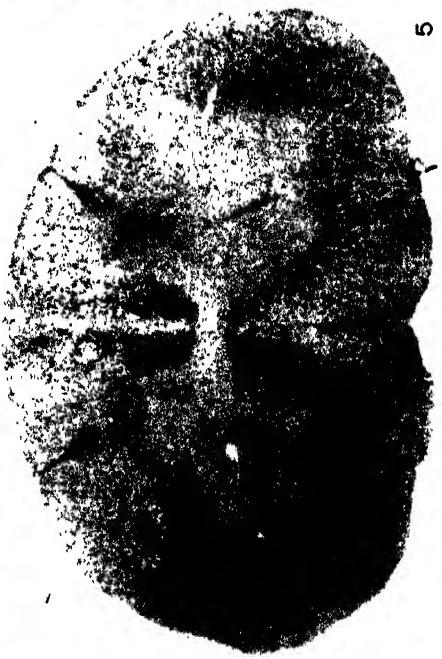
# THE INFLUENCE OF ALUMINUM SULFATE AND ALUMINUM HYDROXIDE UPON THE ABSORPTION OF DIETARY PHOSPHORUS BY THE RAT [1]

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The work of previous investigators has indicated that the feeding of sufficient amounts of soluble aluminum salts under experimental conditions renders much of the phosphorus of the ration non-assimilable. Thus, Cox, Dodds, Wigman and Murphy ('31) have reported that, if aluminum is added to the diets of rabbits or guinea pigs in quantities slightly in excess of that necessary to react with all the phosphorus contained in the ration (as $AlPO_4$), the absorption of phosphorus is practically nil. Deobald and Elvehjem ('35) have described similar results with chickens, using a natural grain ration to which various levels of aluminum salts were added. The addition of aluminum sulfate in amount equivalent to the phosphorus content of the ration resulted in severe symptoms of rickets, with low levels of blood inorganic phosphate. Likewise, Jones ('38) has described the production of marked rickets with low blood inorganic phosphate in rats fed a stock ration with high levels of aluminum sulfate or basic aluminum acetate.

The current use of aluminum hydroxide in the treatment of gastrointestinal disturbances such as peptic ulcer and

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[1] This paper was presented in preliminary form at the meeting of the American Physiological Society in Chicago, April 16, 1941 (Street and Barlow).

gastritis has suggested a re-investigation of the effect of aluminum compounds upon phosphorus utilization.

Since this study was completed it has been reported by Fauley and co-workers ('41) that the administration of aluminum hydroxide gel to dogs in amounts equal to twice the phosphorus content of the diet reduced the urinary excretion of inorganic phosphate to approximately half its normal value.

The work reviewed above does not give precise quantitative data as to the effect of aluminum on phosphorus utilization since the degree of availability of the phosphorus in the cereal diets used is unknown (Jones, '39). The experiments described in this paper were designed to determine more quantitatively the influence of aluminum on phosphorus absorption.

### METHOD

It was decided to study the utilization of phosphorus by means of the growth rate of young rats placed on a diet adequate except for phosphorus. The addition of sodium phosphate and aluminum compounds in various amounts and ratios to such a diet presumably should permit an accurate evaluation of the effect of aluminum on phosphorus utilization.

The low phosphorus diet of Schneider and Steenbock ('39), slightly modified, was chosen as the most suitable for the purposes desired. By adding phosphate to a diet of this type, which is composed of purified food materials, rations can be constructed in which the phosphorus is present chiefly in known chemical form in contra-distinction to the form and uncertain availability of the phosphorus in the cereal rations formerly used in studies of phosphorus utilization.

The percentage composition of the diet used was as follows: sucrose 44, starch 20, rice bran concentrate [2] 4, dried egg white 18, phosphorus-free salt mixture 4, and cottonseed oil 10. To this was added 0.3 cc. of a 0.3% solution of carotene in oil,

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[2] Vitab rice bran concentrate, obtained from the National Oil Products Company, Inc., Harrison, New Jersey.

400 I.U. of vitamin D (viosterol), and 400 µg. of riboflavin per 100 gm. of ration.

The phosphorus content of this ration was reported by Schneider and Steenbock as 0.04%. Their data indicate that young rats placed on the basal ration alone grow very poorly, but the simple addition of sufficient sodium acid phosphate to bring the phosphorus content of the ration to 0.27% completely corrects the deficiency and results in satisfactory growth.

Various dietary formulae were developed from this basal ration by partially replacing the sucrose with sodium acid phosphate, or with both phosphate and aluminum sulfate or hydroxide in definite molecular proportions of aluminum to phosphorus. The phosphorus content of the basal ration was determined by the method of Fiske and Subbarow ('25) after digestion of the food by a wet ashing technique. Various lots of the basal diet were found to contain 0.035 to 0.042% phosphorus; the egg white used as the source of protein in the ration was found to have a phosphorus content of 0.08 to 0.10%. The drinking water used was a spring water of negligible phosphorus content.

The solubility of aluminum hydroxide in dilute acid and, therefore, in the stomach, depends upon its method of preparation. U. S. P. aluminum hydroxide is practically insoluble in tenth-normal hydrochloric acid. This material should be physiologically inert as a component of the diet, since it would not dissolve in the stomach. On the other hand, certain forms of commercial aluminum hydroxide used in clinical medicine are soluble in dilute hydrochloric acid.

The aluminum hydroxide used in this study was a powdered commercial tablet mixture.[3] Its solubility in acid was indicated by the fact that, when placed in tenth-normal hydrochloric acid, it was found to combine with and neutralize 75% of the theoretical amount of acid within a 20-minute period.

[3] Creamalin tablets, obtained from the Alba Pharmaceutical Company, Inc., Rensselaer, New York.

Young rats weighing 40 to 50 gm. were placed on experimental rations in groups of ten. The growth rates were determined at weekly intervals over a 6-week period, after which the animals were sacrificed and the blood inorganic phosphate determined individually by the method of Fiske and Subbarow ('25).

The massed results of feeding three series of rations are shown below in experiments I to III.

*Experiment I.* In this experiment different groups of animals were fed the basal low-phosphorus ration alone, or the basal ration plus sodium acid phosphate, or phosphate plus aluminum hydroxide, as indicated in table 1.

It will be seen that the animals on the basal ration grew at the rate of about 3 gm. per week, while the rats of groups 2 and 3, with phosphorus levels of 0.24% and 0.60%, respectively, gained approximately 17 gm. per week. With groups 4 and 5 fed a diet containing 0.6% and 3.0% $Al(OH)_3$, respectively, sufficient phosphate was added to the ration to make the value of the atomic ratio of aluminum to phosphorus equal to 1. In other words, the quantity of aluminum present was that calculated as just sufficient to precipitate all the phosphorus as $AlPO_4$, assuming complete interaction. On the basis of the growth curves the percentile formation of insoluble aluminum phosphate which actually occurred was much less than theory, since the animals of both groups 4 and 5 grew at approximately two-thirds the rate of those in groups 2 and 3 receiving optimum phosphorus.

It will be seen that the observed levels of the blood inorganic phosphate of the different groups were approximately proportional to the growth.

The somewhat subnormal growth of the positive controls in this experiment was due to the use of unheated egg white in the ration, thus resulting in early symptoms of egg white damage (Parsons and Kelly, '33). This artifact was proven by two following experiments in which the dietary egg white

was steamed, dried and ground, thus precluding the possibility of egg white damage.

It is clear that the effect of the aluminum feeding on phosphorus absorption in this experiment was much smaller than would be expected from the work of previous investigators. The two following experiments were carried out in an effort to explain this discrepancy.

TABLE 1

*Summary of experiments I, II and III.*

Experiment I

| GROUP NO | MATERIAL ADDED TO BASAL DIET | PHOSPHORUS CONTENT OF RATION | ATOMIC RATIO Al : P | AVERAGE GAIN IN WEIGHT IN 6 WEEKS | BLOOD INORGANIC PHOSPHORUS, AVER. VALUE |
|---|---|---|---|---|---|
| | | % | | gm | mg /100 cc |
| 1 | None | 0.04 | | 19.8 | 3.7 |
| 2 | $NaH_2PO_4 \cdot H_2O$ | 0.24 | | 105.6 | 8.2 |
| 3 | $NaH_2PO_4 \cdot H_2O$ | 0.60 | | 100.6 | 9.0 |
| 4 | $NaH_2PO_4 \cdot H_2O$ + 0.6% $Al(OH)_3$ | 0.24 | 1:1 | 70.6 | 6.0 |
| 5 | $NaH_2PO_4 \cdot H_2O$ + 3.0% $Al(OH)_3$ | 1.20 | 1:1 | 72.3 | |

Experiment II

| 1 | None | 0.04 | | 11.1 | 3.54 |
|---|---|---|---|---|---|
| 2 | $NaH_2PO_4 \cdot H_2O$ | 0.20 | | 128.9 | 7.15 |
| 3 | $NaH_2PO_4 \cdot H_2O$ | 0.60 | | 145.2 | 8.37 |
| 4 | $NaH_2PO_4 \cdot H_2O$ + 1% $Al(OH)_3$ | 0.20 | 2:1 | 43.3 | 3.19 |
| 5 | $NaH_2PO_4 \cdot H_2O$ + 1% $Al(OH)_3$ | 0.60 | 2:3 | 137.7 | 7.87 |
| 6 | $NaH_2PO_4 \cdot H_2O$ + 2.58% $Al_2(SO_4)_3 \cdot$ 18 $H_2O$ | 0.24 | 1:1 | 11.9 | 3.66 |
| 7 | $NaH_2PO_4 \cdot H_2O$ + 2.58% $Al_2(SO_4)_3 \cdot$ 18 $H_2O$ | 0.60 | 2:5 | 142.4 | 9.00 |

Experiment III

| 1 | None | 0.04 | | 4.7 | 2.40 |
|---|---|---|---|---|---|
| 2 | $NaH_2PO_4 \cdot H_2O$ | 0.10 | | 38.4 | 2.98 |
| 3 | $NaH_2PO_4 \cdot H_2O$ | 0.15 | | 95.9 | 4.46 |
| 4 | $NaH_2PO_4 \cdot H_2O$ | 0.20 | | 140.8 | 4.88 |
| 5 | $NaH_2PO_4 \cdot H_2O$ + 1% $Al(OH)_3$ | 0.20 | 2:1 | 46.8 | 2.98 |
| 6 | $NaH_2PO_4 \cdot H_2O$ + 1% $Al(OH)_3$ | 0.60 | 2:3 | 161.7 | 7.08 |
| 7 | $NaH_2PO_4 \cdot H_2O$ + 0.5% $Al(OH)_3$ | 0.20 | 1:1 | 84.2 | 3.65 |
| 8 | $NaH_2PO_4 \cdot H_2O$ + 2.58% $Al_2(SO_4)_3 \cdot$ 18 $H_2O$ | 0.24 | 1:1 | 17.7 | 2.27 |

*Experiment II.* In this experiment, summarized in table 1, the effect of aluminum sulfate on phosphorus utilization was compared with that of aluminum hydroxide. The growth of groups 2 and 3 indicates that the level of 0.20% phosphorus in the ration is slightly limiting, since a diet containing 0.60% phosphorus permitted a significantly greater growth. The incompleteness of combination of the ingested aluminum hydroxide with phosphate is indicated by the growth of group 4. Since the gain in weight of this group was one-third that of group 2, receiving the slightly limiting level of 0.20 gm. phosphorus per 100 gm. of ration, it may be assumed that the amount of phosphorus remaining available in ration 4 was approximately one-third that of group 2, or 0.067 gm. per 100 gm., indicating that 0.133 gm. of phosphorus per 100 gm. of ration combined with aluminum. Since sufficient aluminum was present to combine with 0.400 gm. phosphorus per 100 gm., the extent of the combination was only one-third of theory. The performance of group 5 indicates that this limiting effect of 1% aluminum hydroxide on growth can be completely eliminated by increasing the phosphorus level to 0.60%.

The data for group 6 show that when a soluble aluminum salt is fed in an amount just equal to the phosphorus content, there is almost complete failure of growth, indicating that most of the phosphorus in the ration has been made unavailable. Here again, the inhibiting effect of aluminum on growth can be prevented merely by increasing the phosphorus content of the ration, since group 7, receiving 0.60% phosphorus in addition to the aluminum sulfate, made optimum growth.

All three groups of rats showing maximal growth, that is, groups 3, 5 and 7, appeared in excellent condition, with smooth, sleek coats, despite the considerable amount of aluminum in the rations of groups 5 and 7. Thus, the only obviously harmful effect of aluminum feeding in these experiments was its interference with phosphorus utilization.

As in experiment I, the blood inorganic phosphate was low in the rats whose growth was seriously limited by the lack of sufficient available phosphorus, while the groups making normal growth exhibited normal levels of blood phosphate.

*Experiment III.* The results of feeding this series of rations are shown in table 1. This experiment was carried out in order to determine more quantitatively the effect of aluminum hydroxide on phosphorus utilization. From the data of the first four groups, a curve was constructed relating the per cent of available phosphorus in the ration to the growth obtained. Applying this curve to the performance of group 5, receiving 1% aluminum hydroxide, indicates that there was 0.108% available phosphorus in the ration, so that 0.092 gm. of phosphorus per 100 gm. of food, or 46% of the phosphorus present, was rendered unavailable. Since there was sufficient aluminum present to combine with twice the amount of phosphorus actually contained in the ration, it would appear that only 23% of the aluminum was changed to a soluble form so that it could combine with phosphate. Making the same calculation for group 7, the growth attained corresponds to 0.14% of available phosphorus, indicating that 0.06 gm. of phosphorus per 100 gm. of food was rendered unavailable. This is 30% of the phosphorus present in the ration. Since the amount of aluminum present was just sufficient to combine theoretically with all the phosphorus present, this indicates that 30% of the aluminum was converted to a reactive form.

The results obtained with the feeding of aluminum sulfate in this series were similar to those in experiment II. As in preceding experiments, the level of inorganic blood phosphate was approximately proportional to the growth obtained on the various rations.

## DISCUSSION

These experiments indicate that when a soluble form of aluminum, as aluminum sulfate, is fed to young rats in amount equal to the phosphorus content of the ration, there is nearly complete precipitation of phosphorus in the intestinal tract.

This conclusion would appear justifiable, since the growth and levels of blood inorganic phosphate obtained under these circumstances are little, if any, greater than those on the basal low phosphorus ration alone. Since aluminum hydroxide is very insoluble in neutral solution, the ability of this form of aluminum to react with phosphate in the intestinal tract is undoubtedly due to its solution by the acid gastric contents, forming aluminum chloride. It follows, then, that the much smaller effect of the hydroxide on phosphorus absorption, as compared to the sulfate, indicates that only a portion of the aluminum hydroxide dissolves in the stomach.

## SUMMARY

When aluminum sulfate was fed in amounts chemically equivalent to the phosphorus in the ration, essentially all of the phosphorus was rendered unavailable. On the other hand, when aluminum hydroxide was fed to young rats at levels of 0.5 and 1.0% of the diet about one-third to one-fourth of the aluminum was converted to a form reacting with phosphorus.[4]

## LITERATURE CITED

AMERICAN MEDICAL ASSOCIATION COUNCIL ON PHARMACY AND CHEMISTRY 1941 New and nonofficial remedies: Aluminum hydroxide gel. J. Am. Med. Assoc., vol. 117, p. 1539.

Cox, G. J., M. L. DODDS, H. B. WIGMAN AND F. J. MURPHY 1931 The effects of high doses of aluminum and iron on phosphorus metabolism. J. Biol. Chem., vol. 92, p. xi.

[4] Dick and Eisele ('42) have recently referred to our preliminary report (Street and Barlow, '41) as the basis in part for their statement, "The use of aluminum hydroxide and aluminum sulfate, which recently has become quite widespread, may have deleterious effects. Therapeutic doses may interfere with the absorption of inorganic phosphate from the intestinal tract, thereby deranging the calcium metabolism of the body." This statement is misleading. Aluminum sulfate is not, to our knowledge, used medicinally. The maximal therapeutic dose of aluminum hydroxide gel by normal oral administration recommended by the A.M.A. Council on Pharmacy and Chemistry ('41) would supply not more than 64 cc. in 24 hours. This amount of 5.5% gel would furnish 3.66 gm. of $Al(OH)_3$. If 30% of this were changed to soluble form as indicated by our experimental data it would combine with 0.44 gm. of P, since 1 gm. of aluminum hydroxide is equivalent to 0.4 gm. P. If a dietary contains 1.32 gm. of P, the level recommended by Sherman ('37), the precipitation of 0.44 gm. P leaves 0.88 gm. available, which is the figure given by Sherman as the minimal maintenance level for a 70 kg. adult. Thus, our data do not support the conclusions drawn from them by Dick and Eisele.

DEOBALD, H. J., AND C. A. ELVEHJEM 1935 The effect of feeding high amounts of soluble iron and aluminum salts. Am. J. Physiol., vol. 111, p. 118.

DICK, G. F., AND C. W. EISELE 1942 Peptic ulcer. J. Am. Med. Assoc., vol. 118, p. 38.

FAULEY, G. B., S. FREEMAN, A. C. IVY, A. J. ATKINSON AND H. S. WIGODSKY 1941 Aluminum phosphate in the therapy of peptic ulcer. Arch. Int. Med., vol. 67, p. 563.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

JONES, J. H. 1938 The metabolism of calcium and phosphorus as influenced by the addition to the diet of salts of metals which form insoluble phosphates. Am. J. Physiol., vol. 124, p. 230.

——— 1939 A comparison of cereal and non-cereal diets in the production of rickets. J. Nutrition, vol. 18, p. 507.

PARSONS, H. T., AND E. KELLY 1933 The effect of heating egg white on certain characteristic pellagra-like manifestations produced in rats by its dietary use. Am. J. Physiol., vol. 104, p. 150.

SCHNEIDER, H., AND H. STEENBOCK 1939 A low phosphorus diet and the response of rats to vitamin $D_2$. J. Biol. Chem., vol. 128, p. 159.

SHERMAN, H. C. 1937 Chemistry of Food and Nutrition, Fifth Edition. The Macmillan Company, New York, N. Y.

STREET, H. R., AND O. W. BARLOW 1941 The relative effects of aluminum hydroxide and aluminum sulfate on the absorption of dietary phosphorus by the rat. Am. J. Physiol., vol. 133, p. 465.

# THE ASCORBIC ACID CONTENT OF EWES' BLOOD, COLOSTRUM AND MILK AND THE EFFECT OF ASCORBIC ACID INJECTIONS [1]

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TWO FIGURES

The ascorbic acid content of ewes' milk is of interest with respect to both human nutrition and the physiology of vitamin C. Ewes' milk, although not extensively used as a food in the United States, is of great importance as an article of human diet both in European countries and on the continents of Asia and Africa. The total annual world production for human consumption, as given by Zorn et al. ('38) is 5032 million liters. Investigations by these same authors of the nutritive value of ewes' milk for infant feeding indicate that normal development results when infants are weaned on ewes' milk or transferred from cows' milk to ewes' milk. Investigations by the Sudbury Laboratory ('40) indicate that ewes' milk can be profitably produced by American farmers.

Rasmussen, Bogart and Maynard ('38) report values ranging from 25 to 40 mg. per quart (2.7–4.2 mg. per 100 ml.) for the ascorbic acid content of ewes' milk, obtained from determinations on samples taken from one ewe at intervals over a 9-week period. Zorn, Richter and Wiener ('38) give 3.18 mg. per 100 ml. as the average value for the ascorbic acid content

of milk samples taken from five or six East Friesian milk ewes during 16 weeks in 1936 and 23 weeks in 1937. Buruiana ('39) obtained the value 109.6 mg. per liter (10.96 mg. per 100 ml.) using methylene blue titration.

In the present paper results are reported of the determination of the ascorbic acid content of 104 samples of ewes' blood, and of 165 samples of colostrum and milk by titration with 2,6-dichlorophenolindophenol, and the effect of injections of ascorbic acid on the content of the milk and blood.

### EXPERIMENTAL PROCEDURE

Twenty bred Hampshire ewes were used. From June through November, 1938, they were on lespedeza and grass pasture. From December 5, 1938, to April 24, 1939, the daily ration per ewe consisted of 4 lbs. of soybean hay or soybean hay and whole soybeans. After lambing, in addition to the hay, a concentrate mixture made up of 2 parts corn, 2 oats, 2 wheat bran, and 1 part cottonseed meal was fed at the rate of 1.5 lbs. per head daily.

Blood samples were taken at 3- to 4-week intervals during the months of October, 1938, through January, 1939. Sixteen of the twenty ewes lambed and from these ewes samples of colostrum and milk were obtained over a period of about 11 weeks during the months of February through May, 1939. During the last 2 weeks of the period feeding was discontinued and the animals were placed on Italian rye grass and crimson clover pasture.

At the end of this time, while still on pasture, ten ewes received intramuscular injections of ascorbic acid in the scapula region. Five of the animals received injections of 2 gm. each and five received injections of 5 gm. each. Samples of milk were taken at the time of injection, 4 hours after injection, and daily for 5 days following the injection. Blood samples were taken 4 hours after and 1 day after injection. Two other ewes were used as controls, milk and blood samples being taken at the same time as were those from the injected animals.

## ANALYTICAL PROCEDURE

*Blood.* The blood was drawn into 1-oz. blue glass bottles containing 20 mg. of sodium oxalate and 10 mg. of sodium cyanide for each 20 ml. of blood. The ascorbic acid determination was made by the method of Satterfield, Perlzweig and Dann ('37). Four milliliters of plasma, free from hemoglobin pigments, were mixed with 6 ml. of reboiled distilled water and 10 ml. of 4% metaphosphoric acid and filtered. Five milliliter portions of the filtrate were placed in 40 ml. capsule vials and titrated with 2,6-dichlorophenolindophenol using a modified Neale-Forbes titration assembly. The dye solution, previously standardized against a solution of pure ascorbic acid, was added until 1 drop produced a faint pink color visible for 30 seconds. Each titration was completed within 3 minutes.

*Milk.* The colostrum and milk samples were titrated by the method of King ('37). Five milliliters of milk and 2½ ml. of a solution containing 8% acetic acid and 4% metaphosphoric acid were mixed in a 40 ml. capsule vial and titrated until the appearance of a pink color as above.

### RESULTS

The ascorbic acid content of blood for individual ewes before lambing, averaged over the period, ranged from 0.43 to 0.82 mg. per 100 ml. of plasma (table 1) with an average value of 0.66 for eighty determinations. Wide variations were observed in the ascorbic acid levels of blood samples from the same ewe taken at the different sampling periods. Values for single samples range from a low of 0.31 to a high of 1.18.

Comparison of these values, 0.43–0.82 mg. per 100 ml., with values reported for other species shows them to be approximately the same as those for the goat, 0.6–0.8, reported by Richmond et al. ('40), and but slightly lower than those for the human on an adequate vitamin C intake, 0.75 or above, reported by Abt, Farmer and Epstein ('36). Wallis ('40) gives the somewhat lower value of 0.320 for cows' blood, and Holmes, Tripp and Satterfield ('39) find the averages for several groups of Rhode Island Red hens to have the some-

what higher values of from 1.061 to 1.906 mg. per 100 ml. of blood plasma.

The ascorbic acid content of colostrum was found to be considerably higher than that of milk. The values for samples of colostrum (table 1) obtained before the lamb was allowed to suckle ranged from 2.01 to 9.94 mg. per 100 ml., the average value being 5.87. The values were found to drop rapidly during the first few days after lambing. After the fifth or sixth day the rapid decrease in ascorbic acid concentration ceased and from this point throughout the remainder of the period of observation, irregular variations were found. The average values for the milk of individual ewes from the sixth day until the end of the period ranged from 0.38 to 1.77 mg. per 100 ml., with an average of 0.80 mg. per 100 ml. (table 1).

This value is considerably lower than the values 2.7–4.2 mg. per 100 ml. (Rasmussen, Bogart and Maynard, '38), 3.18 (Zorn, Richter and Wiener, '38), and 10.96 (Buruiana, '39) reported by others. It should be noted that the animals used were not milk ewes. Also, though there is not full agreement as to the effect of diet on the ascorbic acid content of milk, it should be pointed out that the diet of these animals contained no good source of vitamin C. This value, however, is not greatly different from the values reported for goats' milk by Richmond et al. ('40) of 0.5–2.0. It is somewhat lower than the values of Holmes, Tripp, Woelffer and Satterfield ('40) for cows' milk: 1.776–2.337 for Guernsey and 1.572–2.044 for Holstein.

It is seen that the average ascorbic acid content of colostrum obtained before the lamb suckled was roughly seven times that of milk obtained after the sixth day, and that the ascorbic acid content dropped rapidly during the first 5 or 6 days to a relatively constant value. Figure 1 shows the general trend for the ascorbic acid content of ewes' colostrum and milk. The average curve, fitted by inspection, is given with two individual curves superimposed as an indication of the variation from this normal average curve.

A high value for the ascorbic acid content of cows' colostrum as compared with that of milk was reported by Rasmussen et al. ('36). These authors believed this to indicate a certain storage of ascorbic acid by the cow during the preparturition period. Kon and Watson ('37) found cows' colostrum to contain slightly more ascorbic acid than did milk. Selleg and King ('36) found that the ascorbic acid content
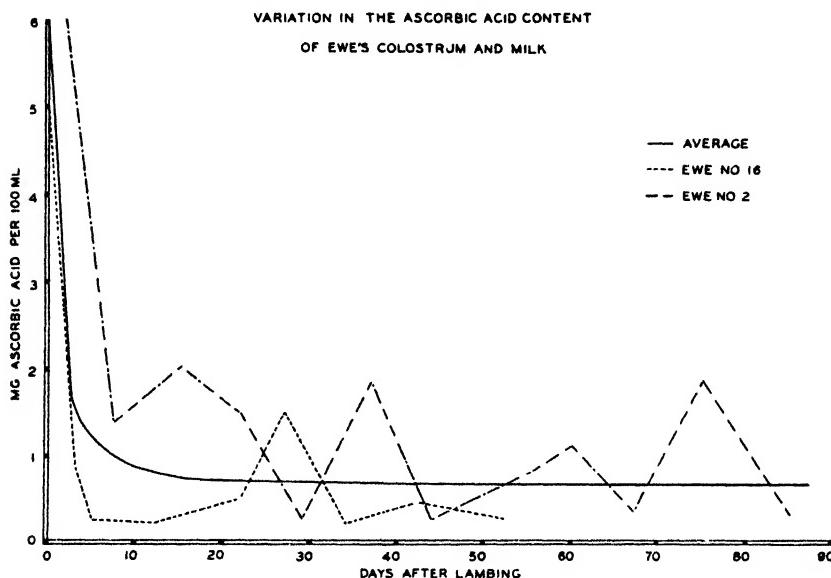


Figure 1

of human colostrum, based upon twenty cases, showed wide variation, but gave a value that was comparable with normal milk. Kasahara and Kawashima ('36) report that the ascorbic acid content of colostrum of Japanese women is 2 to 4 times that of normal breast milk, slowly falling to normal after 3 weeks.

The average values for the ascorbic acid content of blood, colostrum, and milk for each of the animals used is given in table 1.

The twelve animals used in the determination of the effect of injection of ascorbic acid were divided into three groups.

TABLE 1

*Ascorbic acid content of ewes' blood, colostrum and milk.*

| EWE NO. | ASCORBIC ACID IN MG. PER 100 ML. IN | | | EWE NO. | ASCORBIC ACID IN MG. PER 100 ML. IN | | |
|---|---|---|---|---|---|---|---|
| | Blood [1] | Colostrum [2] | Milk [3] | | Blood | Colostrum | Milk |
| 2 | 0.67 | 7.73 | 1.05 (11) | 35 | 0.66 | ...[5] | ... |
| 3 | 0.65 | 8.87 | 1.09 (12) | 36 | 0.59 | 7.26 | 0.51 (4) |
| 8 | 0.66 | 7.71 | 0.82 (11) | 37 | 0.52 | 7.70 | 0.72 (9) |
| 9 | 0.76 | 2.01 | 0.66 (12) | 41 | 0.64 | ...[5] | ... |
| 13 | 0.55 | 4.04 | 0.93 (9) | 42 | 0.68 | ...[5] | ... |
| 15 | 0.61 | ...[4] | 0.68 (9) | 45 | 0.71 | ...[5] | ... |
| 16 | 0.82 | 5.01 | 0.49 (7) | 46 | 0.62 | 6.08 | 0.68 (2) |
| 18 | 0.74 | 5.00 | 0.54 (10) | 49 | 0.79 | 4.62 | 1.77 (19) |
| 28 | 0.70 | 3.06 | 1.06 (10) | 51 | 0.43 | 3.05 | 0.54 (7) |
| 29 | 0.81 | 5.99 | 0.94 (11) | 982 | 0.66 | 9.94 | 0.38 (9) |

[1] Average of four samples taken at 20–25 day intervals, prior to lambing.

[2] Sample collected before lamb was allowed to suckle.

[3] Average of samples obtained after 6th day from lambing. Figures in parentheses indicate number of duplicate determinations included in average.

[4] Colostrum nursed out before sample could be taken.

[5] Did not lamb.

Group I was composed of two animals which received no injections, group II was composed of five animals each of which received an injection of 2 gm. of ascorbic acid, and group III was composed of five animals each of which received an injection of 5 gm. of ascorbic acid. In the group receiving 2-gm. injections the ascorbic acid content of blood samples taken 4 hours after injection was roughly 4 times the normal value, ranging from 2.87 to 3.13; in the group receiving 5-gm. injections, it was roughly 10 times the normal, ranging from 5.95 to 7.93. After 1 day the blood content of animals of both groups had dropped to approximately normal values of from 0.84 to 1.11.

The effect of the injections on the ascorbic acid content of the milk is shown in figure 2. As a result of large variations between animals within groups, a larger number of animals would be required to give more definite conclusions. Individual averages for animals in group II varied from 3.07 to 6.01, and in group III from 3.27 to 6.79 mg. per

100 ml. The increase after injection for individuals varied from 11 to 30% of the initial values in group II and from 15 to 50% of the initial values in group III. The daily group averages which are plotted in figure 2 indicate a fairly rapid rise in the ascorbic acid content of the milk during the first 24 hours after injection and a return to approximately the initial level after 3 days. The average increases of groups II and III were 17.6 and 20.8%, respectively, of the initial average value.



EFFECT OF INJECTION OF ASCORBIC ACID ON THE ASCORBIC ACID CONTENT OF MILK
TWO MONTHS AFTER EWES WERE TURNED ON PASTURE
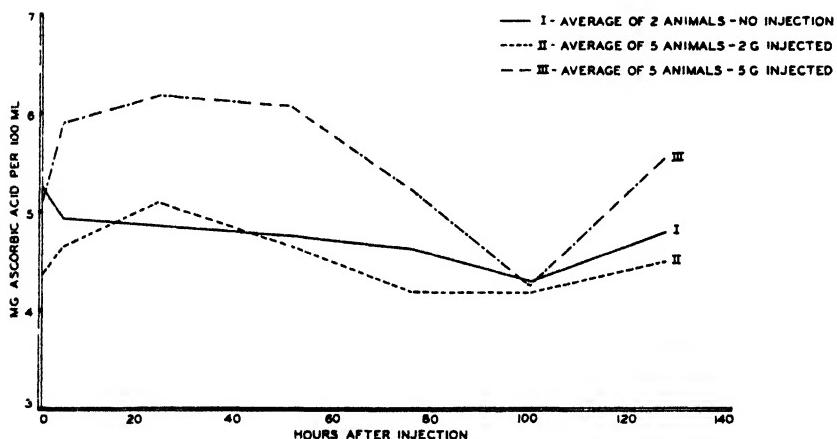
Figure 2

There have been conflicting reports as to the effect of ascorbic acid injections on the concentration of the vitamin in the milk. Sharp ('36) found injections to have no effect on the milk ascorbic acid for the goat; Wendt ('38) noted the same for the cow. On the other hand Richmond et al. ('40) report a small rise in the ascorbic acid content of goats' milk following intraperitoneal injection of 1 or 2 gm. of ascorbic acid. Rasmussen, Bogart and Maynard ('38) report a rise in the ascorbic acid content of milk of one ewe and also of two cows following injections. The present study indicates that injection of ascorbic acid, in this case intramuscularly,

results in a rise in the ascorbic acid content of ewes' milk and a return to normal within 3 days. The magnitude of this rise, however, varies widely with different individuals.

## SUMMARY

Results are given of the determination of the ascorbic acid content of 104 samples of ewes' blood and of 165 samples of ewes' colostrum and milk by titration with 2,6-dichlorophenolindophenol, and the effect of injections of ascorbic acid on the content of the milk and blood.

The ascorbic acid content of ewes' blood was found to be 0.43–0.82 mg. per 100 ml. of plasma.

The value for the ascorbic acid content of colostrum obtained before the lamb suckled was found to range from 2.01 to 9.94 mg. per 100 ml. The values dropped rapidly during the first 5 or 6 days to the relatively constant value of 0.80 mg. per 100 ml. for milk.

Injection of ascorbic acid resulted in a rise in the ascorbic acid level in the blood and milk. The level in the blood returned to practically normal within 1 day and the level in the milk within 3 days. There was wide variation in the magnitude of the rise in milk ascorbic acid in different animals.

### LITERATURE CITED

ABT, A. F., C. J. FARMER AND I. M. EPSTEIN 1936 Normal cevitamic (ascorbic) acid determinations in blood plasma and their relationship to capillary resistance. J. Pediatrics, vol. 8, p. 1.

BURUIANA, L. 1939 Le taux de l'acid ascorbique dans le lait de quelques mammiferes. Lait, vol. 19, p. 449.

HOLMES, A. D., FRANCIS TRIPP AND G. HOWARD SATTERFIELD 1939 The ascorbic acid content of blood plasma of laying hens. Poultry Science, vol. 18, p. 192.

HOLMES, A. D., FRANCIS TRIPP, E. A. WOELFFER AND G. HOWARD SATTERFIELD
1940  Influence of age of cow on ascorbic acid content of certified milk.
Food Research, vol. 5, p. 263.

KASAHARA, M., AND K. KAWASHIMA  1936  The vitamin C content of colostrum.
Klin. Wochschr., vol. 15, p. 1278.

KING, C. G.  1937  Personal communication to G. Howard Satterfield.

KON, S. K., AND M. B. WATSON  1937  The vitamin C content of cows' milk.
Biochem. J., vol. 31, p. 223.

RASMUSSEN, RUSSEL, RALPH BOGART AND L. A. MAYNARD  1938  Ascorbic acid
content of milk of various species as influenced by ascorbic acid in-
jection and diet. Proc. Soc. Exp. Biol. and Med., vol. 3, p. 502.

RASMUSSEN, RUSSEL, N. B. GUERRANT, A. O. SHAW, R. C. WELCH AND S. I.
BECHDEL  1936  The effects of breed characteristics and stages of
lactation on the vitamin C (ascorbic acid) content of cow's milk.
J. Nutrition, vol. 11, p. 425.

RICHMOND, MARTHA S., G. HOWARD SATTERFIELD, C. D. GRINNELLS AND W. J. DANN
1940  Ascorbic acid content of goat's milk and blood: influence of
ascorbic acid injection and diet. J. Nutrition, vol. 20, p. 99.

SATTERFIELD, G. HOWARD, W. A. PERLZWEIG AND W. J. DANN  1937  Unpublished
data, Duke University.

SELLEG, IVA, AND C. G. KING  1936  The vitamin C content of human milk and
its variation with diet. J. Nutrition, vol. 11, p. 599.

SHARP, PAUL F.  1936  Vitamin C in pasteurized milk. Science, vol. 84, p. 461.

SUDBURY LABORATORY  1940  Personal communication to G. Howard Satterfield.

WALLIS, G. C.  1940  Vitamin C in the nutrition of dairy cattle. J. Dairy
Science, vol. 23, p. 569.

WENDT, C. V. (WITH W. LOJANDER AND W. EHRSTRÖM)  1938  Studies of vitamin
C in cow's milk. Skand. Arch. Physiol., vol. 80, p. 398.

ZORN, W., F. RICHTER AND C. WIENER  1938  Die Bedeutung der Schafmilch für
die Volksernährung. Mit besonderer Berücksichtigung ihrer Eignung
für die Säuglingsernährung. Ernährung, vol. 3, p. 85. Nutr. Abs. and
Revs., vol. 8, p. 462.

# THIAMINE CLEARANCE AS AN INDEX OF NUTRITIONAL STATUS [1]

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Studies of the urinary excretion of thiamine by normal subjects before and after taking test doses, factors influencing such excretion and the changes associated with experimental deficiency have been reported from this laboratory (Melnick, Field and Robinson, '39). On the basis of this work standards were set forth for the interpretation of the urinary thiamine values in the diagnosis of thiamine deficiency. The diagnostic value of such analyses was confirmed by a study of the urinary excretion of thiamine in a large number of clinical cases (Robinson, Melnick and Field, '40).

In the present investigation there is reported a simpler and more reliable procedure for the detection of thiamine deficiency which lends itself readily to routine clinical use.

## EXPERIMENTAL PART

Thirty-seven male and female subjects were used in this study. None of these had disorders which might be expected to lead to either faulty absorption, storage, utilization or excretion of the vitamin. Twenty-three were members of the hospital staff and subsisted regularly on what are generally

regarded as adequate diets. These were considered to be the normal subjects. Fourteen patients having clinical signs or symptoms of thiamine deficiency and giving dietary histories of inadequate thiamine intake constituted the deficient group.

During the 3 days of urinary collections each subject subsisted on a constant diet furnishing at least 900 µg. of thiamine daily. On the first day the basal 24-hour urine sample was collected. On the second day, 12 hours following the last meal, a 4-hour urine sample was obtained from each of the subjects. This is referred to in the present report as the fasting urine sample. Immediately after this period, each subject received parenterally 350 µg. of thiamine per square meter of body area [3] (Peters and Van Slyke, '31). The subsequent 4-hour urine sample was then collected. During these two urinary collection periods, the subjects were in the fasting state. The basal diet was then administered. On the following day and immediately after the largest (midday) meal, a dose of 5000 µg. of thiamine was taken orally.[4] The following 24-hour urine sample was collected. All the urine specimens were then tested for thiamine content according to the colorimetric method of Melnick and Field ('39).

Results of the study are presented in figures 1 to 4. In figure 1 the basal 24-hour urinary thiamine value obtained with each subject is plotted against the per cent of the oral test dose excreted.

In general the correlation is good, confirming our previous reports (Melnick, Field and Robinson, '39; Robinson, Melnick and Field, '40). The deficient subjects excreted small amounts of thiamine, when diet alone was the sole source of the vitamin, and subsequently small fractions of the oral test dose. The

[3] The thiamine solution, containing 500 µg. per cubic centimeter, was administered intramuscularly through a no. 24 needle. Minimal to moderate pressure was applied after the injection.

[4] The necessity for observing these details in collecting 24-hour urine samples, before and after the administration of the oral test dose, was demonstrated by the fundamental studies reported from this laboratory (Melnick, Field and Robinson, '39). Otherwise, variable urinary thiamine values may be obtained which are impossible to interpret.
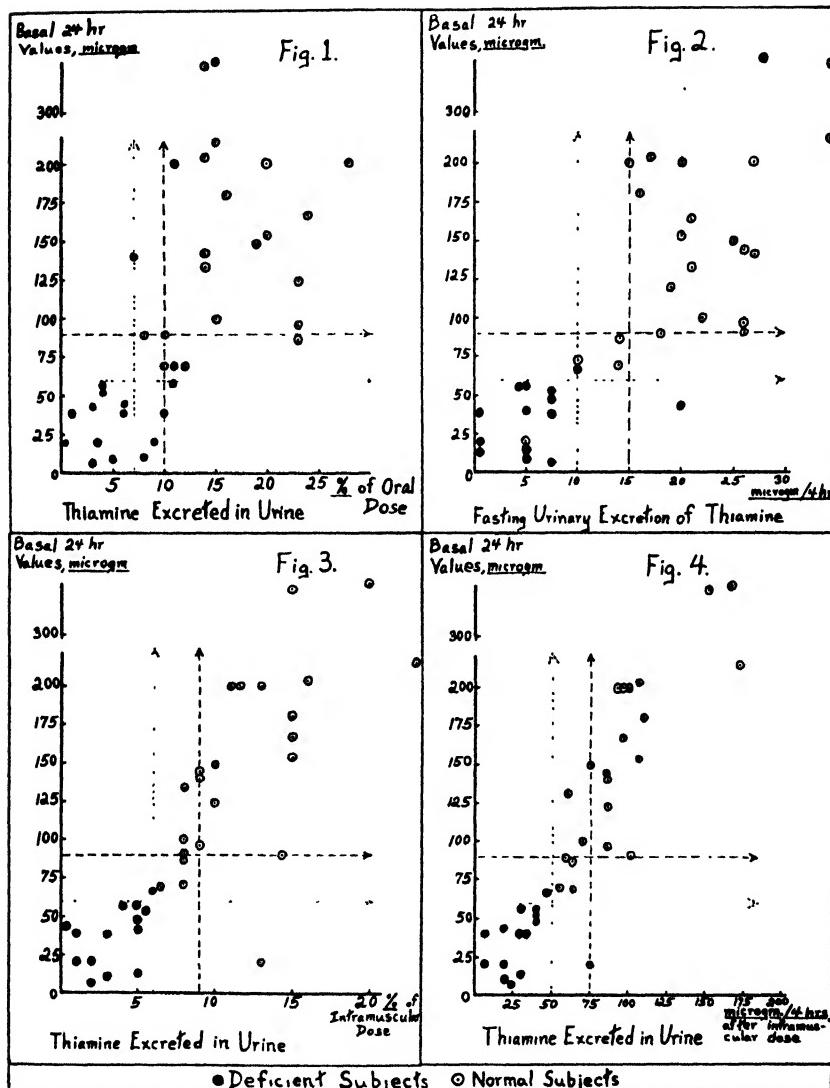
Figs. 1–4   Correlation between the basal 24-hour urinary thiamine value and the response to the oral test dose of extra thiamine (fig. 1), the 4-hour fasting urinary excretion value (fig. 2), the response to the intramuscular test dose (fig. 3) and the total thiamine excreted during the 4-hour period following intramuscular administration of the vitamin (fig. 4).

normal subjects excreted larger quantities of thiamine in both cases. On each of the graphs (figs. 1 through 4) two additional horizontal and vertical ordinates have been drawn. Those in dotted lines represent what we regard, from both our present and previous studies, as the excretion levels below which thiamine deficiency is invariably found. The ordinates drawn in broken lines represent the bottom limits of the normal urinary thiamine excretion. This level of thiamine excretion is about 50% greater than the basal minimal standards indicated by the dotted lines. The area between the dotted and the broken lines may be regarded as the critical range with respect to thiamine deficiency. This interpretation is supported by the fact that subjects subsisting on diets suboptimal in thiamine intake were shown to fall (Robinson, Melnick and Field, '40) precisely in this range of urinary excretion of thiamine.

It will be noted (fig. 1) that thirteen of the fourteen deficient subjects excreted less than 60 µg. of thiamine in their basal urine samples and that ten of the fourteen excreted less than 7% of the oral test dose. Only one of the twenty-three normal individuals excreted less than the 60 µg. quantity of thiamine in the basal sample. All of the deficient subjects and four of the normal group excreted less than 90 µg. of thiamine in the basal sample. Three of the deficient subjects and twenty of the normal subjects excreted 10% or more of the oral test dose.

In figure 2, the fasting 4-hour urinary excretion of thiamine is plotted against the basal 24-hour values obtained on the previous day. The level of thiamine excretion during the fasting period, associated with deficiency, is considered to be 10 µg. or less; the minimal normal level, 15 µg., 50% greater. The distribution of subjects following this test was found to be comparable to that noted in figure 1. Twelve of the fourteen deficient subjects excreted less than 10 µg. during the fasting 4-hour period; only one of the twenty-three normal subjects excreted in this low range. One deficient subject excreted more than 15 µg. in the fasting sample, while nineteen of the normal subjects excreted in excess of this border-line level.

In figure 3 the per cent of the intramuscular test dose of thiamine excreted in the urine during the first 4-hour period is plotted against the basal urinary thiamine values. The level of excretion, associated with thiamine deficiency, was found to be 6% or less of the test dose. The border line is considered to be 9% of the dose, 50% greater than the minimal level of excretion. Thirteen of the fourteen deficient subjects excreted less than 6% of the intramuscular test dose; none of the normal subjects excreted such a small fraction. All of the deficient subjects excreted less than 9% of the dose. Six of the normal subjects excreted less than this quantity after parenteral administration of the vitamin.

In figure 4 no correction has been made for the fasting urinary excretion of thiamine after the administration of the intramuscular test dose. The total thiamine excretion, in micrograms per 4-hour period, is plotted against the basal 24-hour urinary thiamine value. A distinct difference between the responses of normal and deficient groups is noted. Thus, for the routine diagnosis of thiamine deficiency, it should not be necessary to determine the basal fasting urinary thiamine value. Deficient subjects in the fasting state consistently excrete small amounts of thiamine as well as a small fraction of the test dose. Thus, the over-all effect, as plotted in figure 4, parallels closely the results obtained when the responses, before and after administration of the parenteral test dose, are plotted separately. All of the normal subjects excreted a total quantity of thiamine in excess of 50 µg. during the 4-hour test period after dosage; none of the deficient individuals excreted as much. Six of the twenty-three normal subjects (26%) excreted less than 75 µg. of thiamine. This value, which is 50% above the minimal level of excretion, as in the case of the other graphs, is considered to be critical.

## DISCUSSION

No attempt was made in the present study to differentiate between sexes. It has previously been shown that the proportion of parenterally administered (available) thiamine,

excreted in the urine by the normal subject, increases with increasing dosage. This attempt to conserve thiamine when the supply is limited is probably the principal factor responsible for the smaller percentage excretion of ingested vitamin by the normal women subjects (Melnick, Field and Robinson, '39). Additional studies in our laboratory have indicated that when the women subjects ingested diets similar to those eaten by the males, comparable urinary values are obtained. Accordingly, the proposed standards (Melnick, Field and Robinson, '39) for the detection of thiamine deficiency in the case of the female subjects should be revised to coincide with those established for the male. To accept this interpretation, one must conclude that the female subjects whom we considered to be normal were subsisting on borderline levels of thiamine intake. For this group the average daily thiamine intake was approximately 700 µg. At this level of intake the lower urinary excretion indicates an attempt on the part of the body to conserve its supply of thiamine (Melnick, '42).

Confirming an earlier report (Robinson, Melnick and Field, '40), the extent of the urinary excretion of thiamine before and after oral administration of a test dose (5 mg.) was found in the present study to be a reliable index of the nutritional status with respect to this vitamin. Previously (Melnick, Field and Robinson, '39), a preference was indicated for the oral route for administration of the test dose ' of thiamine. Under such circumstances the rate of excretion is much slower than when the same dose is given by any of the parenteral routes. The organism is rapidly "flooded" with the vitamin after parenteral administration so that the major excretion after dosage in such cases occurs within the first 4-hour period. Such a flooding effect is noted even in patients with obvious thiamine deficiency, and to such an extent that the expected difference in excretion between such individuals and normals is masked. However, in the earlier report comparisons were drawn when 5 mg. doses were administered through the various routes. In the present study, the flooding

effect following intramuscular injection of thiamine was practically eliminated by administration of a small test dose, 0.35 mg. per square meter of body area. (This dose is equivalent to approximately 0.65 mg. for the average male subject.)

There are many advantages in using the response to the parenteral test dose of thiamine both for the diagnosis of thiamine deficiency and for conducting prolonged studies of thiamine metabolism. Errors so commonly encountered in the clinic in the routine collection of 24-hour urine samples are eliminated. It is now necessary simply to collect a 4-hour sample, and this lends itself readily to direct supervision. The parenteral route eliminates variations in rate and degree of intestinal absorption of the vitamin encountered when the oral test dose is taken. The test dose is administered by the clinician and its quantitative intake is not dependent upon cooperation of the patient — a potential source of error.

With this simplified test, it is possible to study "outpatients" during a relatively short visit to the clinic and even to conduct, with a good degree of reliability, field surveys of the extent of thiamine deficiency.

The repeated use of the oral test dose in prolonged studies of thiamine metabolism is in itself a form of therapy. Accordingly, proper interpretation of the results becomes difficult. By occasional use of the small parenteral test dose, averaging a total of 650 µg. of the vitamin, the possibility of significant therapeutic effects being produced is almost eliminated. The omission of the major meal on the day when the parenteral test dose is administered serves to counteract the minimal therapeutic value of the dose so that the nutritional status of the subject during the prolonged study is not significantly changed. Furthermore, the major portion of the small parenteral test dose is excreted within the first 4-hour period so that no carry-over effects are noted.

## SUMMARY

Four different thiamine clearance tests were conducted on each of thirty-seven normal and deficient adult subjects.

These consisted of the measurement of the basal 24-hour urinary excretion of thiamine, the fasting 4-hour excretion of the vitamin, the response to the oral administration of 5 mg. of thiamine and the 4-hour excretion of the vitamin when 350 μg. per square meter of body area are administered parenterally. Plotting the results of these tests, one against the other, gave good correlation. The numerous advantages in the use of the parenteral test dose procedure make it the method of choice for studying clinical cases. All normal subjects, but none of the deficient individuals, excreted in excess of 50 μg. of total thiamine during the 4-hour period following dosage.

## LITERATURE CITED

MELNICK, D.  1942  Vitamin B₁ (thiamine) requirement of man. J. Nutrition, vol. 24, pp. 139–152.

MELNICK, D., AND H. FIELD, JR.  1939  Chemical determination, stability and form of thiamine in urine. J. Biol. Chem., vol. 130, pp. 97–107.

MELNICK, D., H. FIELD, JR., AND W. D. ROBINSON  1939  A quantitative chemical study of the urinary excretion of thiamine by normal individuals. J. Nutrition, vol. 18, pp. 593–610.

ROBINSON, W. D., D. MELNICK AND H. FIELD, JR.  1940  Urinary excretion of thiamine in clinical cases and the value of such analyses in the diagnosis of thiamine deficiency. J. Clin. Invest., vol. 19, pp. 399–408.

PETERS, J. P., AND D. D. VAN SLYKE  1931  Quantitative Clinical Chemistry, vol. I (1264 pp.), p. 21, The Williams and Wilkins Co., Baltimore.

# VITAMIN B₁ (THIAMINE) REQUIREMENT OF MAN

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THREE FIGURES

Cowgill ('38) has reviewed the literature up to 1938 dealing with the human requirements for vitamin $B_1$.

The synthesis of thiamine was soon followed by the publication of chemical and microbiological methods for its determination. Reports from many laboratories using biological assay methods (Harris and Leong, '36; Aoki, '39), the colorimetric procedure (Melnick, Field and Robinson, '39; Hou and Yang, '39; Robinson, Melnick and Field, '40; Cahill, '41; Melnick and Field, '42), the thiochrome method (Westenbrink and Goudsmit, '37; Wang and Yudkin, '40; Najjar and Holt, '40; Carden, Province and Ferrebee, '40; Williams, Mason, Wilder and Smith, '40), and the yeast fermentation procedure (Pollack, Dolger, Ellenberg and Cohen, '40; Youmans, Paton, Kennedy, Monroe and Moore, '40; Pollack, Ellenberg and Dolger, '41 a, b) have shown that the extent of urinary excretion of thiamine is a good index of the nutritional status of the human subject.

The present report is the first attempt through objective thiamine balance studies conducted with humans to determine the vitamin $B_1$ requirement and the incidence of inadequate

thiamine intake among so-called normal subjects. Some of the material reported herein has appeared in detail elsewhere (Melnick, Field and Robinson, '39; Robinson, Melnick and Field, '40; Melnick and Field, '42) but the implications of the data relative to the thiamine requirement of man were not emphasized.

The colorimetric method of Melnick and Field ('39) was used throughout this investigation for the estimation of urinary thiamine.

More than 175 normal and deficient subjects were used in the urinary excretion studies carried out by the author and his associates. Of this group 116 had no disorders which might be expected to lead to either faulty absorption, storage, utilization or excretion of the vitamin. These persons were used in the thiamine excretion studies summarized in this report. Sixty of the latter individuals, composed of hospital staff members, laboratory technicians and obviously well-nourished hospital patients constituted the normal group. The remaining fifty-six subjects were patients who exhibited clinical signs or symptoms of thiamine deficiency and gave dietary histories of inadequate thiamine intake.

The results obtained in studies (Robinson, Melnick and Field, '40) of the urinary excretion of thiamine by thirty-nine male and thirty-three female subjects subsisting on adequate diets on the days of the test have indicated good correlation between the urinary excretion of thiamine and the adequacy' of the dietary regime prior to conducting the tests. The normal individual consuming an adequate diet apparently tends to excrete part of the extra dietary thiamine in the urine. The deficient subject attempts to conserve dietary thiamine to replenish depleted tissue stores, and not waste it by urinary excretion. When a test dose of thiamine was superimposed upon the dietary intake, the extra vitamin was handled in the same manner as that obtained from the diet, in that it was conserved or wasted, depending upon the nutritional status of the subject. Confirmatory evidence obtained with thirty-seven more subjects has been presented in the preceding

paper (Melnick and Field, '42). In all of these studies the
normal subjects subsisted on diets furnishing on the average
of 1.0 mg. (333 International Units) of thiamine per day.

This tendency of the depleted subject to conserve thiamine
is observed in both clinical and experimental thiamine de-
ficiency. The addition of thiamine to the inadequate diet to
raise the level of intake to that of the normal group (in the
neighborhood of 1 mg.), does not effect a parallel increase
in the urinary thiamine excretion for a considerable period
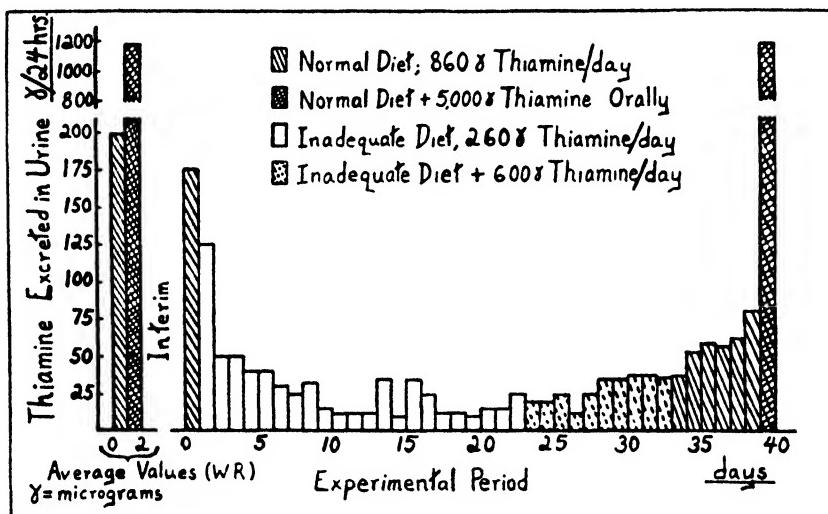


Fig. 1  Urinary excretion of thiamine by the normal individual subsisting on an
inadequate thiamine diet and then on the same ration with added vitamin.

of time (Melnick, Field and Robinson, '39; Robinson, Melnick
and Field, '40). This is best illustrated by the results plotted
in figure 1.

One of the normal subjects, who had been carefully standard-
ized with respect to urinary thiamine excretion while on a
constant diet furnishing 860 μg. of thiamine daily, was then fed
a low thiamine diet for an extended period. All elements of
the diet except thiamine were kept constant. The daily thiamine
intake during the depletion period was 260 μg. After the
twenty-second day the diet was supplemented with enough

thiamine to equal that of the normal basal ration. The vitamin supplement in aqueous solution was partitioned so that the total thiamine intake at each meal was exactly the same as when the normal diet was ingested.

It will be observed (fig. 1) that the urinary thiamine decreased precipitously from the normal to levels characteristic of avitaminotic individuals. On the twenty-third day, when vitamin supplementation was begun, the body stores of thiamine were significantly reduced. Actually there was apparent definite clinical evidence of deficiency, characterized by aching and tenderness of the calf muscle and Achilles tendon, and paresthesia of the lower extremities. Low thiamine excretion values persisted despite supplementation of the diet with the vitamin.

In order to show that there were no unknown factors associated with the ingestion of the inadequate diet tending to augment the thiamine requirements, the subject returned to his normal basal ration. The urinary thiamine values remained in the subnormal range, indicating that the subject was still conserving dietary thiamine to replenish depleted stores. The percentage of the oral dose excreted in the urine at the end of the study, however, rose to the original normal values. Apparently the thiamine reserves of this individual had been restored by the time the oral test dose was administered.

The difference in thiamine content between the normal and deficient diets in the above study was 600 µg. The addition of that quantity of thiamine to the deficient ration, when clinical symptoms of deficiency were apparent, was without appreciable effect upon the excretion values. However, in the normal subject, such an increment in thiamine intake is promptly followed by an increase in the urinary value of about 100 µg. Analyses of thirty 24-hour urine samples collected over a period of 8 months from two normal male subjects,[2] ingesting adequate diets of varying thiamine content, gave urinary thiamine values paralleling the dietary intakes.

[2] One of these subjects was the same individual used in the study of the urinary excretion of the vitamin during experimental thiamine deficiency.

In one individual the excretion varied from 120 to 220 µg., and in another it ranged from 120 to 260 µg. of thiamine. The range in thiamine intake for the two subjects was from 800 to 1400 µg. per day. Removal of the extra thiamine from the diets of these normal subjects resulted in a return to the basal excretion value within 24 hours. The prompt response of the normal subjects to variations in thiamine intake occurred while they were subsisting on their usual diets furnishing approximately 1 mg. of thiamine per day. Apparently a
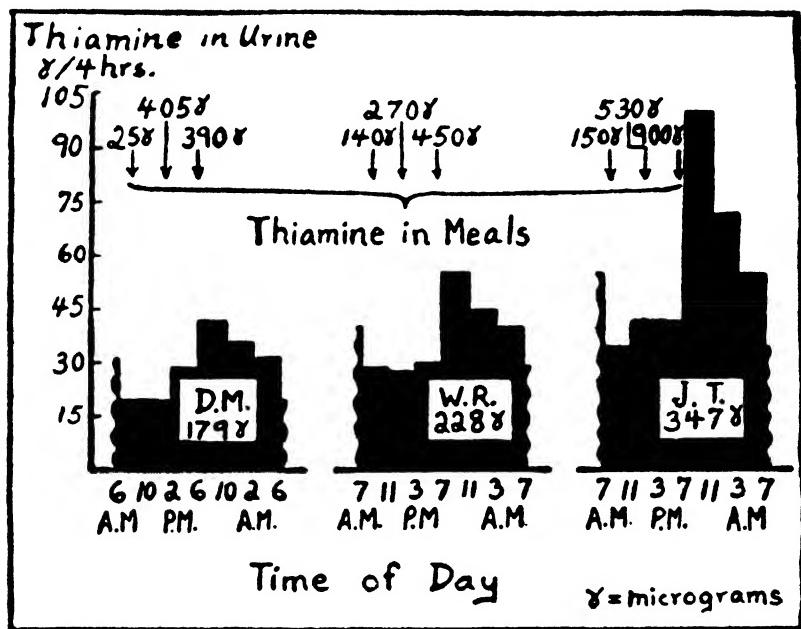


Fig. 2   Normal diurnal variation in urinary thiamine values.

constant daily intake of 1 mg. is sufficient to saturate the subject to such an extent that there is no necessity to conserve extra dietary thiamine.

This conclusion is supported by individual analyses of the six consecutive 4-hour urine samples excreted during a 24-hour period, illustrated graphically in figure 2. A diurnal variation in thiamine excretion is noted in these normal subjects, the extra thiamine being excreted promptly so that by morning

basal minimal figures are obtained. In cases of thiamine deficiency, however, there is no diurnal variation in excretion of the vitamin. The values are minimal and constant.

Subsequent investigations of the urinary excretion of thiamine by normal subjects before, during, and after obvious thiamine saturation have yielded additional evidence in support of the conclusion that a dietary intake of approximately 1 mg. of thiamine per day is adequate for satisfying at least

TABLE 1

*Urinary excretion of thiamine by the normal adult before and after thiamine saturation.*

| SUBJECT | SURFACE AREA | CUSTOMARY DAILY THIAMINE INTAKE | URINARY EXCRETION OF THIAMINE,[1] µG./24 HRS | | | | |
|---------|--------------|-------------------------------|--------|--------------------------------------|-----------------------------------|--------|-------------------------------------|
| | | | Basal | Increment after oral test dose [2] | Increment during saturation [3] | "Basal" | Increment after oral test dose [2] |
| | *sq.m* | *µg.* | | | | | |
| D. C. | 1.60 | 870 | 130 | 950 | 4170 | 339 | 825 |
| F. O. | 1.64 | 950 | 150 | 750 | 4170 | 269 | 650 |
| J. C. | 1.75 | 840 | 138 | 1025 | 5230 | 337 | 1000 |
| D. M. | 1.88 | 1060 | 208 | 925 | 4080 | 370 | 775 |
| M. H. | 1.96 | 1370 | 125 | 1040 | 4420 | 308 | 775 |
| L. S. | 2.18 | 1630 | 118 | 700 | 4000 | 276 | 650 |
| Average | 1.84 | 1120 | 145 | 898 | 4350 | 317 | 779 |

[1] On the day that urine samples were collected, each subject received a well-balanced diet furnishing 1070 µg. of thiamine per 2700 calories.

[2] Five milligrams of thiamine taken orally after dinner.

[3] Ten milligrams of thiamine were taken orally each day, 3.33 mg. after each meal, for a period of from 3 to 6 weeks.

the minimal requirement of the adult. The more important features of the study are presented in table 1.

On each of the days of urinary collections the individuals were fed the same constant adequate diet furnishing in each case 1070 µg. of thiamine.[3] The 24-hour urinary thiamine

[3] The thiamine values for the other diets used in the present study were based upon the tables compiled by Williams and Spies ('38). In this particular case, the foods were frozen in solid carbon dioxide immediately after cooking, ground to a uniform mixture and aliquots taken for thiamine analyses according to a modification of the thiochrome method of Hennessy ('41). The figure obtained agreed with that calculated from the tables of Williams and Spies ('38). This gives added support to the reliability of the thiamine values estimated for the remaining diets.

values for the six subjects before and after the ingestion of an oral dose of 5 mg. of the vitamin were all well within the normal range (Melnick, Field and Robinson, '39; Robinson, Melnick and Field, '40; Melnick and Field, '42). During a 3 to 6 weeks' period of thiamine saturation 10 mg. of extra thiamine were ingested daily, 3.33 mg. after each meal. The 24-hour urine samples were collected at the end of each week. Within 1 week the subjects were excreting more than 4 mg. of thiamine in excess of their basal output. This indicates that the organism can excrete large fractions (40 to 50%) of dietary thiamine if a sufficient excess is available. During the remaining period of vitamin saturation, the urinary values remained relatively constant at this high level of excretion. When dosage with extra thiamine was stopped, a precipitous drop in the 24-hour urinary excretion values occurred within the first 4 days to approximately 400 µg. followed by further gradual decrease. Previous tests (Melnick, Field and Robinson, '39) have shown that fully 2 weeks are required before the 24-hour urinary values return to the previous basal level. In the present study, however, the subjects were given the oral dose of 5 mg. of thiamine 7 days after termination of the saturation period. The high "basal" excretions during the 24 hours prior to the administration of the oral test dose constitute good evidence that the six subjects were still in a state of thiamine saturation. The use of this "basal" value in determining the increment of thiamine excretion after the oral test dose was justified since at this period following saturation the return to the normal level is very gradual. The total urinary thiamine figures following administration of the test dose to the saturated subjects were slightly higher than those obtained prior to saturation. The greater values, however, were due entirely to the higher "basal" values noted during this test period. After corrections were made for these elevated "basal" figures, the values were found to be no greater than those obtained in the initial thiamine clearance tests. Because of the possibility that the urinary excretion of extra thiamine following its oral administration to a subject in the saturated state might be slower than that noted in the

first thiamine clearance test, analyses were made of the urine collected during the second 24-hour period following dosage. These showed the same small carry-over values as were observed in the initial tests; viz., 1 to 2% of the oral test dose. Apparently, the thiamine stores of these normal subjects subsisting regularly on diets furnishing approximately 1 mg. of thiamine daily were in no significant state of depletion prior to the saturation regime. Otherwise, a greater degree of thiamine clearance, following administration of the oral dose, should have occurred during the latter test when the subjects were known to be in a state of thiamine saturation. That this deduction is valid is indicated by similar tests conducted on subjects who subsisted previously on diets low in thiamine. The thiamine clearance values were much greater (sixfold) when the tests were conducted after the period of thiamine saturation (Robinson, Melnick and Field, '40).

In a previous study [4] (Melnick, Field and Robinson, '39) on the excretion of thiamine by normal subjects, the average 24-hour urinary value for the males was found to be approximately 200 µg.; the dietary intake was about 1000 µg. In the case of the normal women used in this study, the average daily urinary excretion was approximately 90 µg. The dietary intake, however, was not proportionately less, but in the neighborhood of 700 µg. This is considered to be good evidence that when the dietary intake is 700 µg., there is an attempt on the part of the organism to conserve its thiamine intake and not waste it by urinary excretion. The 700 µg. intake of thiamine may then be regarded as border-line with respect to the normal daily requirement. Support for this is indicated by the fact that when both the normal men and women are furnished extra thiamine (as the oral test dose) they excreted approximately the same fraction, about 13%, of this additional thiamine. In other words, the women were not sufficiently deficient, despite their low thiamine intake,

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[4] In this particular investigation the normal subjects, male and female, subsisted on their customary, supposedly adequate, diets of their own choosing.

to show evidence of conservation of the thiamine when an excess became available.

It is of interest to point out that these normal subjects on diets furnishing from 2000 to 3000 calories daily required from 700 to 1000 µg. of thiamine before the above objective signs of conservation of dietary thiamine were apparent. Calculations based upon Cowgill's formula ('34), derived in studies conducted before synthetic thiamine became available, indicate that a dietary vitamin to calorie ratio of 2.0 (or a thiamine to calorie ratio [5] of 0.3) should protect against symptoms of beriberi. Such a vitamin to calorie ratio is synonymous with a dietary intake of 600 to 900 µg. for individuals subsisting on 2000 to 3000 calories per day. This is in excellent agreement with the critical 700 to 1000 µg. range, derived from the urinary excretion studies. The values based upon Cowgill's formula should be expected to be slightly less, since his criterion was protection against the appearance of frank deficiency symptoms.

The value derived from the urinary excretion studies of approximately 350 µg. of thiamine per 1000 calories may be regarded as the minimal daily requirement. If, as is generally done in drawing up other nutritional standards, a 50% margin of safety is allowed, the recommended daily intake becomes approximately 500 µg. per 1000 calories. The validity of these minimal and recommended values for thiamine intake is also supported by the studies of Keys and Henschel ('42), which indicated no improvement, following thiamine supplementation, in the physiological and biochemical responses of active adult males subsisting regularly on diets furnishing 430 µg. of the vitamin per 1000 calories.

As a corollary to the present report, it is of interest to estimate how many of the so-called normal subjects actually subsist upon inadequate thiamine intakes. Figure 3 presents a summation of the results of the studies conducted with the

[5] One Cowgill vitamin unit, the milligram equivalent, is equal to 0.15 µg. of thiamine.

116 subjects at the University Hospital, Ann Arbor, and at the Food Research Laboratories.

These subjects are first classified as deficient, border-line, or normal on the basis of clinical signs, symptoms, and dietary histories. The responses of these subjects to the four types of urinary excretion tests are also indicated. Examination



Fig. 3    Correlation between thiamine clearance tests and diagnoses of nutritional status, based upon clinical examination.

of the findings demonstrates that there is a tendency for the clinician to be conservative in his classifications. This is undoubtedly due to the difficulty in detecting sub-clinical or latent signs of thiamine deficiency even by those specially trained in the diagnosis of the avitaminoses. Subjects clinically regarded as deficient, were invariably found to be so according

to results obtained by the four types of laboratory tests; those classified as border-line showed a tendency to excrete minimal quantities of thiamine characteristic of the deficient group, while normal subjects frequently excreted border-line, or even lower quantities of thiamine.

The data used in figure 6 were reassembled in table 2, since not all the subjects were used in all the tests. Some, classified as normal, gave border-line urinary values in more than one type of test; others in only one. For purposes of determining adequacy of nutrition, the normal individual should be regarded as one who passes all of the laboratory tests.

TABLE 2

*Nutritional status of subjects used in the present study.*

| NUMBER OF SUBJECTS | CLINICAL CLASSIFICATION | INTERPRETATION OF CLEARANCE TESTS | | | |
| --- | --- | --- | --- | --- | --- |
| | | Malnourished | | Normal | |
| | | Number | Per cent | Number | Per cent |
| 34 | Deficient | 34 | 100 | 0 | 0 |
| 16 | Border-line | 14 | 87 | 2 | 13 |
| 66 | Normal | 18 | 27 | 48 | 73 |

The results presented in table 2 indicate that the urinary thiamine values of all subjects classified as deficient by clinical criteria were well below the critical range of excretion. Only two of the sixteen border-line cases fell within the normal range in both types of laboratory tests to which they were subjected. Fully eighteen of the sixty-six normal subjects gave low thiamine clearance values in one or more of these tests, as though they were border-line or deficient subjects. Thus, only 73% of the normal subjects were excreting well enough in the normal range to pass all four criteria. This is a surprisingly low percentage inasmuch as these normal subjects consisted mainly of staff members of the hospital or of laboratory personnel whose diets were in no way restricted. However, this high incidence of failures among our so-called normal subjects to pass all the clearance tests may be ex-

plained by the recent observation of Lane, Johnson and Williams ('42) that the average American diet, prior to the advent of enriched bread and flour, furnished only 320 μg. of thiamine per 1000 calories.

## SUMMARY

On the basis of objective thiamine balance studies, the vitamin $B_1$ requirement of the adult is estimated to be 350 μg. per 1000 calories. The recommended daily intake is regarded as 500 μg. per 1000 calories. Only 73% of the so-called normal subjects, who were not restricted as to choice of diet, excreted sufficient quantities of thiamine in the urine to pass all the thiamine clearance tests.

## LITERATURE CITED

AOKI, S. 1939 Studies on the vitamin $B_1$ in human urine. Japan J. Med. Sci. VIII, Internal Med., Pediat., Psychiat., vol. 5, proc. p. 167.

CAHILL, W. H. 1941 Urinary excretion of thiamine on high-fat and high-carbohydrate diets. J. Nutrition, vol. 21, pp. 411–418.

CARDEN, G. A., W. D. PROVINCE AND J. W. FERREBEE 1940 Clinical experiences with the measurement of the urinary excretion of vitamin $B_1$. Proc. Soc. Exp. Biol. Med., vol. 45, pp. 1–5.

COWGILL, G. R. 1934 The Vitamin $B_1$ Requirement of Man, 265 pp., Yale University Press, New Haven.

————— 1938 Human requirements for vitamin $B_1$. J. Am. Med. Assoc., vol. 111, pp. 1009–1016.

HARRIS, L. J., AND P. C. LEONG 1936 Vitamins in human nutrition. The excretion of vitamin $B_1$ in human urine and its dependence on the dietary intake. Lancet, vol. 1, pp. 886–895.

HENNESSY, D. J. 1941 Chemical methods for determination of vitamin $B_1$. Ind. Eng. Chem., Anal. Ed., vol. 13, pp. 216–218.

HOU, H. C., AND E. F. YANG 1939 Observations on vitamin $B_1$ metabolism. I. Urinary excretions by normal individuals and beriberi patients. Chinese J. Physiol., vol. 14, pp. 269–282.

KEYS, A., AND A. F. HENSCHEL 1942 Vitamin supplementation of U. S. Army ration in relation to fatigue and the ability to do muscular work. J. Nutrition, vol. 23, pp. 259–269.

LANE, R. L., E. JOHNSON AND R. R. WILLIAMS 1942 Studies of the average American diet. I. Thiamine content. J. Nutrition, vol. 23, pp. 613–624.

MELNICK, D., AND H. FIELD, JR. 1939 The chemical determination, stability and form of thiamine in urine. J. Biol. Chem., vol. 130, pp. 97–107.

————— 1942 Thiamine clearance as an index of nutrition. J. Nutrition, vol. 24, pp. 131–138.

MELNICK, D., H. FIELD, JR., AND W. D. ROBINSON 1939 A quantitative chemical study of the urinary excretion of thiamine by normal individuals. J. Nutrition, vol. 18, pp. 593–610.

NAJJAR, V. A., AND L. E. HOLT, JR. 1940 Studies in thiamine excretion. Bull. Johns Hopkins Hosp., vol. 67, pp. 107–124.

POLLACK, H., H. DOLGER, M. ELLENBERG AND S. COHEN 1940 A test proposed to measure vitamin $B_1$ saturation in human subjects. Proc. Soc. Exp. Biol. Med., vol. 44, pp. 98–100.

POLLACK, H., M. ELLENBERG AND H. DOLGER 1941 a Clinical studies on vitamin $B_1$ excretion determined by the fermentation method. Arch. Int. Med., vol. 67, pp. 793–804.

———————— 1941 b Excretion of thiamine and its degradation products in man. Proc. Soc. Exp. Biol. Med., vol. 47, pp. 414–417.

ROBINSON, W. D., D. MELNICK AND H. FIELD, JR. 1940 Urinary excretion of thiamine in clinical cases and the value of such analyses in the diagnosis of thiamine deficiency. J. Clin. Invest., vol. 19, pp. 399–408.

WANG, Y. L., AND J. YUDKIN 1940 Assessment of the level of nutrition. Urinary excretion of aneurin at varying levels of intake. Biochem. J., vol. 34, pp. 343–352.

WESTENBRINK, H. G. K., AND J. GOUDSMIT 1937 Relation of the uptake and the excretion of aneurin (vitamin $B_1$) in the case of normal subjects. Arch. néerland. physiol., vol. 22, pp. 319–325.

WILLIAMS, R. D., H. L. MASON, R. M. WILDER AND P. F. SMITH 1940 Observation on induced thiamine (vitamin $B_1$) deficiency in man. Arch. Int. Med., vol. 66, pp. 785–799.

WILLIAMS, R. R., AND T. D. SPIES 1938 Vitamin $B_1$ (thiamin) and its use in medicine, 411 pp., The Macmillan Co., New York.

YOUMANS, J. B., E. W. PATON, A. KENNEDY, S. C. MONROE AND B. MOORE 1940 Vitamin $B_1$ excretion as determined by the fermentation method; its metabolic and clinical significance. Trans. Assoc. Am. Phys., vol. 55, p. 141.

# THE NICOTINIC ACID CONTENT OF MEAT

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The literature now contains a considerable number of observations on the nicotinic acid content of meat; references to these have been collected in the table of Bacharach ('41) and the monograph of Waisman and Elvehjem ('41). Nevertheless, further work is still required, since some of the reported observations are based on analytical methods which are open to criticism (Dann and Handler, '41) and also because most of the figures reported are based on the analysis of a single sample of the particular meat.

In this paper we present the results of analyses of certain commonly-used meats, using samples purchased in local retail stores, together with data on the effect of cooking upon the nicotinic acid content of some of them. The analyses were made by a chemical method previously described (Dann and Handler, '41), in which digests of the samples are treated with Lloyd's reagent and lead hydroxide to decolorize them completely, and nicotinic acid in the colorless extract is then determined by the König reaction using cyanogen bromide and metol. The results are given in micrograms of nicotinic acid per gram fresh weight of meat. In examining the effect of cooking, dry weights of the raw and cooked meats were determined and the nicotinic acid content is expressed on the dry weight basis. This method is subject to error when fat is rendered from the meat during cooking, but the error will be in the direction of increasing the apparent nicotinic acid content of the cooked meat and so decreasing the apparent

loss due to cooking. Therefore, the losses of nicotinic acid from lean meats which lose fat during cooking, such as pork chops, will actually be greater than the figures reported here. The sampling for experiments on the effects of cooking was performed by cutting out a small piece (5 to 10 gm.) of tissue with scissors before cooking and removing a second sample from the tissue close by the first excision after the meat was cooked.

### RESULTS AND DISCUSSION

The data obtained are given in the tables. They may be compared with the considerable series of meats assayed at the University of Wisconsin by three different methods, namely,

TABLE 1

*Nicotinic acid content of meat as purchased. The mean value is accompanied by its standard error when the number of samples is greater than 5.*

| MEAT | NUMBER OF SAMPLES | NICOTINIC ACID PER GRAM FRESH WT. | |
| | | Mean | Range |
|---|---|---|---|
| | | $\mu g$. | $\mu g$. |
| Beef liver | 8 | $100 \pm 7.4$ | 76–143 |
| Beef kidney | 5 | 77 | 65–86 |
| Beef muscle meats | 17 | $57 \pm 3.4$ | 40–82 |
| Lamb kidney | 2 | | 93 and 96 |
| Lamb heart | 6 | $63 \pm 2.2$ | 58–72 |
| Lamb muscle meats | 11 | $77 \pm 3.9$ | 54–102 |
| Pork liver | 11 | $140 \pm 6.5$ | 97–166 |
| Pork muscle meats | 14 | $61 \pm 5.3$ | 41–102 |
| Ham | 11 | $50 \pm 5.7$ | 24–90 |
| Veal liver | 3 | 123 | 115–136 |
| Veal kidney | 4 | 90 | 83–100 |
| Veal muscle meats | 8 | $73 \pm 4.3$ | 49–91 |
| Chicken liver | 15 | $152 \pm 5.2$ | 114–178 |
| leg meat | 15 | $72 \pm 1.8$ | 61–80 |
| breast meat | 15 | $151 \pm 5.7$ | 110–181 |
| Rabbit liver | 2 | | 221 and 215 |
| kidney | 2 | | 130 and 162 |
| leg meat | 2 | | 126 and 129 |
| Trout | 1 | | 35 |
| Halibut | 1 | | 30 |
| Haddock | 1 | | 9 |
| Perch | 1 | | 17 |
| Scallop | 1 | | 14 |

bio-assay with the dog (Waisman et al., '40), chemical deter-
mination by a variant of the König reaction (McIntire et al.,
'41), and microbiological assay with Lactobacillus arabinosus
17-5 (Waisman and Elvehjem, '41). The values obtained with
the Wisconsin samples by microbiological assay all fall within
or just outsides the ranges we have observed. In general the
same samples showed a higher apparent nicotinic acid content
when analyzed by the chemical method employed by McIntire
et al. ('41) and a still higher apparent nicotinic acid content
when the dog bio-assay was performed.

TABLE 2

*Effect of cooking on nicotinic acid content of meats.*

| MEAT AND MODE OF COOKING | NICOTINIC ACID IN MG PER GM OF DRY WEIGHT | | NICO-TINIC ACID LOSS | MEAT AND MODE OF COOKING | NICOTINIC ACID IN MG PER GM OF DRY WEIGHT | | NICO-TINIC ACID LOSS |
|---|---|---|---|---|---|---|---|
| | Uncooked | Cooked | | | Uncooked | Cooked | |
| | | | % | | | | % |
| Chicken leg meat | | | | Pork liver (continued) | | | |
| Frying | 295 | 190 | 36 | Frying | 454 | 366 | 19 |
| Frying | 310 | 157 | 49 | Frying | 450 | 345 | 23 |
| Frying | 309 | 168 | 46 | | | | |
| Frying | 354 | 128 | 64 | Pork chops | | | |
| Roasting | 297 | 189 | 37 | Frying | 172 | 91 | 47 |
| Roasting | 283 | 199 | 30 | Frying | 314 | 136 | 57 |
| Steaming | 320 | 175 | 45 | Frying | 143 | 116 | 19 |
| Steaming | 300 | 163 | 46 | Frying | 155 | 82 | 47 |
| | | | | Frying | 136 | 69 | 49 |
| Chicken breast meat | | | | | | | |
| Frying | 659 | 338 | 49 | Ham | | | |
| Frying | 578 | 328 | 43 | Frying | 143 | 131 | 8 |
| Frying | 438 | 237 | 46 | Frying | 128 | 89 | 30 |
| Frying | 418 | 288 | 31 | Frying | 89 | 76 | 15 |
| Roasting | 585 | 392 | 33 | Frying | 91 | 72 | 21 |
| Roasting | 452 | 336 | 26 | Frying | 170 | 132 | 22 |
| Steaming | 629 | 416 | 34 | Frying | 139 | 111 | 20 |
| Steaming | 642 | 372 | 42 | Frying | 139 | 109 | 22 |
| | | | | Lamb heart | | | |
| Beef liver | | | | Baking | 298 | 123 | 59 |
| Frying | 370 | 222 | 40 | Baking | 270 | 138 | 49 |
| Frying | 314 | 250 | 20 | Baking | 293 | 132 | 55 |
| | | | | Baking | 301 | 98 | 67 |
| Pork liver | | | | Baking | 264 | 114 | 57 |
| Frying | 392 | 290 | 26 | Baking | 279 | 131 | 53 |
| Frying | 536 | 355 | 34 | | | | |

We interpret these observations in the following manner. Agreement between the results of our own chemical method and the microbiological assay has been reported by Snell and Wright ('41) and recently demonstrated by Cheldelin ('42), for a number of foodstuffs analyzed by both procedures. It seems reasonable to assume that close correspondence between results obtained by two such widely different methods indicates that they lead to essentially true values for the nicotinic acid content. In that case the values obtained chemically by McIntire et al. ('41) are too high; and this is in agreement with our previous knowledge of chemical methods for determining nicotinic acid. The method they used was essentially that of Melnick and Field ('40), and earlier observations in this laboratory have shown that incomplete decolorization of the tissue digests by charcoal as used in this method leads to apparent nicotinic acid contents which are higher than the true values.

If this interpretation is correct, the dog bio-assay also gives results which are too high; possibly as has been suggested by Snell and Wright ('41) because the basal diet is incomplete apart from its deficiency of nicotinic acid and the meat supplements act in part by supplying other factors than nicotinic acid.

The results obtained by the bio-assay, being the first to appear, have been widely used in tables showing the nicotinic acid content of foodstuffs, but in the light of this interpretation it now appears that they are too high and that the later lower figures obtained on the same samples by the microbiological assay are more trustworthy.

It will be noticed that muscle meats for each mammalian species have been grouped together. This was done because no evidence of difference in nicotinic acid content of different muscle meats emerged from our figures. Thus the seventeen figures for beef muscle meats include round steak, 50, 51, 58, 58, and 56 µg. per gram; brisket, 58; chuck, 59, 71, and 40; neck, 31; rib, 82, 73, and 66; heart, 81; other cuts, 44, 55, and 44. Possibly rib steak may contain more nicotinic acid than

round steak, but more data would be required to establish this point.

Most of the types of meat show considerable variation from one sample to another. This is no doubt largely due to variations among individuals of the species but may also be partly due to effects of the ageing of the meat, a variable which we have not been able to control. Preliminary experiments with the tissues of rabbits have indicated a definite loss of nicotinic acid after storing the meat for 1 week in a household refrigerator. The possibility of destruction of nicotinic acid during the commercial ageing of meat of other species requires investigation.

The loss of nicotinic acid which we have observed during cooking is surprisingly large. This is at present inexplicable, but important in its application to dietetics. Since most meats are cooked before consumption, only one-half to two-thirds of the nicotinic acid present in the raw meat will generally survive cooking and be ingested; allowances for this must be made when calculating the nicotinic acid content of diets from data obtained by the analysis of raw meats.

Our figures show that the liver of the chicken takes its place with mammalian liver as one of the richest sources of nicotinic acid: it is equalled by the breast meat of the chicken, which is much richer than any other muscle meat we have examined.                                            .

### SUMMARY

Nicotinic acid analyses of a number of meats purchased at retail stores are reported, based on a chemical procedure using completely decolorized digests of the tissue.

The values reported are considerably lower than have been obtained by others using either bio-assay on the dog or a chemical procedure with only partly decolorized digests, but they correspond more closely with results obtained in the microbiological assay and are believed to be more nearly correct.

The effect of cooking on several types of meat has been examined; generally from one-third to one-half of the nicotinic acid was lost, based on the dry weight of the meat.

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## LITERATURE CITED

BACHARACH, A. L. 1941 The distribution of nicotinic acid in human and animal foods. Nutrition Abst. & Rev., vol. 10, p. 459.

CHELDELIN, V. H. 1942 Private communication.

DANN, W. J., AND P. HANDLER 1941 The quantitative estimation of nicotinic acid in animal tissues. J. Biol. Chem., vol. 140, p. 201.

MCINTIRE, J. M., H. A. WAISMAN, L. M. HENDERSON AND C. A. ELVEHJEM 1941 Nicotinic acid content of meat and meat products. J. Nutrition, vol. 22, p. 535.

MELNICK, D., AND H. FIELD, JR. 1940 Determination of nicotinic acid in biological materials by means of photoelectric colorimetry. J. Biol. Chem., vol. 134, p. 1.

SNELL, E. E., AND L. D. WRIGHT 1941 A microbiological method for the determination of nicotinic acid. J. Biol. Chem., vol. 139, p. 675.

WAISMAN, H. A., AND C. A. ELVEHJEM 1941 The vitamin content of meat. Burgess Publishing Co., Minneapolis, Minn.

WAISMAN, H. A., O. MICKELSON, J. M. MCKIBBIN AND C. A. ELVEHJEM 1940 Nicotinic acid potency of food materials and certain chemical compounds. J. Nutrition, vol. 19, p. 483.

# CATARACT AND OTHER OCULAR CHANGES RESULTING FROM TRYPTOPHANE DEFICIENCY [1]

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In the past several years a series of investigations on nutritional cataracts have been made at this laboratory. It has been shown that one type of cataract and keratitis can be obtained readily in the eyes of rats fed riboflavin deficient diets (Day, Langston and O'Brien, '31; Day, Darby and Langston, '37). The discovery by Mitchell and coworkers that cataracts also resulted from feeding of excessive amounts of lactose (Mitchell and Dodge, '34) and galactose (Mitchell, '35) led to a study of other sugars as causative agents. It was reported from here (Darby and Day, '39) that xylose is likewise an effective agent for production of lens opacities. The xylose cataract, which in appearance is identical to that obtained by feeding galactose and lactose, is quite unlike riboflavin deficiency cataract.

The investigations to be reported in this communication were prompted by the observation of Berg and Potgieter [2] that many of their animals on tryptophane-deficient diets developed lens opacities. These workers were not satisfied

that a relationship between the amino acid deficiency and the cataract was sufficiently well-established and they made no comment upon it in their publication (Berg and Potgieter, '32).

A search of the literature revealed that Curtis, Hauge and Kraybill ('32) had recorded the occurrence of what they called "a type of permanent blindness" in rats maintained on a tryptophane-low diet.

<div align="center">EXPERIMENTAL</div>

The compositions of the basic experimental diets are given in table 1. Diet 5000 was similar to the tryptophane-deficient

<div align="center">TABLE 1</div>

<div align="center">*Composition of the amino acid-deficient diets.*</div>

| | DIET NO. | |
| --- | --- | --- |
| | 5000 | 5002 |
| | % | % |
| Acid-hydrolyzed casein ..................... | 14.7 | |
| Cystine ................................... | 0.3 | |
| Whole zein ............................... | | 18.0 |
| Sucrose ............. .................... | 15.0 | 15.0 |
| Starch ................................... | 42.0 | 39.0 |
| Agar ............. ..................... | 2.0 | 2.0 |
| Salt mixture (Hubbell, Mendel & Wakeman, '37). | 2.0 | 2.0 |
| Cod liver oil ............................. | 5.0 | 5.0 |
| Hydrogenated cottonseed oil (Crisco) .......... | 19.0 | 19.0 |

B vitamins furnished by daily supplementation with 250 mg. yeast (Harris).

diet employed by Berg and Potgieter ('32). The acid-hydrolyzed casein was prepared by the method of Berg and Rose ('29). Whole zein [3], which is deficient in both tryptophane and lysine, was used as the chief source of dietary nitrogen in diet 5002.

Litters from two groups of rats were used, one a Wistar strain of albinos and the other a strain of black and white

[3] Purchased from the Prolamine Products Co. of Roby, Indiana, and used without further purification.

hooded animals. Young rats, immediately after weaning (except for two experiments in which older animals were used), were housed in individual wire-bottom cages and given water and the experimental diets ad libitum. Litter-mate controls received 0.1% or 0.2% tryptophane mixed with their diet. All animals were given the B vitamins by daily supplementation with 250 mg. of yeast [4]. Weight, food consumption and ophthalmoscopic records were made at weekly intervals. Slitlamp examinations of the eyes of some of the animals were also made [5].

### RESULTS AND DISCUSSION

The animals on the deficient diets lost weight rapidly and in a few days became emaciated and developed the characteristic hunchback and unkempt appearance typical of tryptophane deficiency in the rat.

Most of the small animals on diet 5000 eventually developed a severe alopecia, particularly on the face. However, the loss of hair was not shown by the animals on the zein diet, nor by the larger animals receiving diet 5000. It is not possible, from our data, to state definitely that the alopecia was due solely to a lack of tryptophane. Diet 5000 has a very oily consistency as contrasted to diet 5002 which is mealy, the zein being bulky enough to absorb much of the fat. Small animals could not easily be kept out of the food containers and the constant contact with the fat of the diet was very probably responsible for the greasy appearance of the hair and may have contributed to the development of the alopecia.

After several weeks on the deficient diets some animals exhibited a nervous manifestation characterized by hyperexcitability, especially to auditory stimuli.

The results of the ophthalmoscopic studies, summarized in table 2, indicate that the lenticular changes observed in the deficient animals are due solely to the lack of tryptophane in

[4] Harris.

[5] We are indebted to Dr. K. W. Cosgrove of the Department of Ophthalmology for the slit-lamp examinations.

TABLE 2

Incidence of cataract and keratitis in rats fed various amino acid deficient diets.

| GROUP NO. | SUPPLEMENT | INITIAL BODY WEIGHT IN | NO. OF ANIMALS DEVELOPING | | | TIME IN DAYS FOR APPEARANCE OF | | |
|---|---|---|---|---|---|---|---|---|
| | | gm. | ophthalmoscopic cataract | gross cataract | keratitis | ophthalmoscopic cataract | gross cataract | keratitis |
| I (14)[1] | 0.2% Tryptophane | 30–48 | 0 | 0 | 0 | .... | .... | .... |
| II (40) | .......... 120 µg. riboflavin per wk. | 30–50 | 37 | 8[2] | 27 | 8–64 | 63–106 | 14–145 |
| III (4) | 0.2% | 32–43 | 4 | 2 | 4 | 18 | 65 | 25–51 |
| IV (6)[3] | Tryptophane 0.1% or 0.2% | 32–36 | 0 | 0 | 0 | .... | .... | .... |
| V (16) | Tryptophane | 26–200 | 0 | 0 | 11 | .... | .... | 11–118 |
| VI (14) | .......... | 26–44 | 12 | 2[4] | 13 | 13–42 | 70–119 | 14–193 |
| VII (6) | .......... | 58–78 | 5 | 1 | 5 | 40–82 | 86 | 11–112 |
| VIII (4) | .......... | 130–210 | 0 | 0 | 3 | .... | .... | 25–46 |

[1] Groups I–IV, inclusive, were fed diet 5000; groups V–VIII, inclusive, received diet 5002. Numbers in parentheses indicate the total number of animals in the group.

[2] Only 21 animals were kept on the experimental diet longer than 63 days.

[3] The diet of these animals was restricted in amount to equal that eaten by deficient animals.

[4] Only 7 animals were kept on the experimental diet longer than 70 days.

their diet. The animals of groups I to IV received the hydrolyzed-casein diet. Those of group I received 0.2% tryptophane mixed with the diet and served as positive controls. All of the animals in this group grew at a good rate and none showed any of the deficiency signs exhibited by the animals of groups II and III. Group II received no tryptophane supplement, and thirty-seven of the forty animals developed ophthalmoscopic cataract in from 8 to 64 days, while twenty-seven also showed vascularity of the cornea. A few had a generalized ophthalmia. Of twenty-one animals in group II which were kept on the diet longer than 63 days, eight developed mature cataracts.

An assay for riboflavin in the yeast which was used as a source of B complex by the method of Darby and Day ('38) showed that it contained 45 µg. per gram. Therefore, the rats in this study each received 68 µg. weekly in the yeast supplement. Inasmuch as it was shown (Day, Darby and Langston, '37) that as little as 30 µg. of riboflavin weekly was adequate to protect against riboflavin deficiency cataract, it seems very unlikely that riboflavin deficiency was involved in the experiments here reported. However, to test this possibility additional amounts of riboflavin equal to 120 µg. weekly were given to the animals of group III. Such supplementation failed to alter significantly either the nature or the time of incidence of the ocular changes.

The results obtained with group IV offer additional proof that a vitamin lack was not responsible for the eye changes seen. The food intake of these animals, receiving diet 5000 supplemented with 0.2% tryptophane, was restricted to equal the intake of litter-mate controls receiving diet 5000 only. Hence, both experimental and control animals received the same quantity of all food factors except tryptophane. None of the group IV animals showed any of the pathological changes seen in groups II and III.

The animals in groups V to VIII received the zein diet which was, of course, deficient in lysine. Those of group V received 0.1% or 0.2% tryptophane mixed with their diet and served

as controls. None of them exhibited cataract but eleven out of sixteen did show vascularization of the cornea. The incidence of keratitis appeared to be the same at either level of tryptophane supplementation. Animals in group VI (deficient diet without supplement) exhibited both cataract and keratitis. Groups VII and VIII were larger animals and received the same diet as group VI. Both groups exhibited keratitis but the group containing the largest animals (VIII) did not show cataract before the termination of the experiment on the eighty-sixth day.

The eye changes seen were both lenticular and corneal, but the corneal manifestations, described below, appeared also in the eyes of many animals receiving a diet containing tryptophane but deficient in lysine. On the other hand, the lack of lysine does not appear to influence the lens changes. However, conclusions regarding specific lesions related to lysine deficiency should not be drawn from results obtained with our experimental regimen since the yeast vitamin supplement undoubtedly contains some of this amino acid. Very slow growth was obtained by use of diet 5002 supplemented with tryptophane, indicating that the amount of lysine received by the animals was not insignificant.

Changes in the lenses of animals on a tryptophane-deficient diet began to appear after 2 to 3 weeks on the experiment. The initial change was an apparent difference in optical density of lens tissue in the form of a concentric band or ring situated in the cortex of the lens. This usually progressed until there was a shell-like appearance of the lens cortex, visible entirely around the lens, both anteriorly and posteriorly. The diameter of this shell appeared to be approximately two-thirds the diameter of the lens. In about one-third of the animals remaining on the diet for a sufficiently long period the lenses became partially or entirely white and opaque. Frequently that part of the lens within the incipient "shell" became opaque, leaving clear lens fibers at the periphery. In other cases the entire lens became opaque. Such mature cataracts were plainly visible to the unaided eye.

The cataract produced by these experimental diets, as seen with the ophthalmoscope, was quite unlike lens opacities resulting from xylose or galactose feeding as well as those resulting from riboflavin deficiency.

Keratitis was observed in the eyes of most of the animals receiving either the tryptophane- or the lysine-deficient diet, as well as in the eyes of those on diets lacking in both amino acids. The most striking change seen was the marked vascularity. Vessels pushed anteriorly from the outer canthus and progressed towards the center until, in some cases, the entire cornea was eventually involved. This vascularity differs from that seen in riboflavin deficient animals in that the vessels in the latter case simultaneously invade the cornea from all parts of the periphery. Also unlike the vascularization resulting from riboflavin deficiency, which frequently occurs only after a diffuse corneal opacity becomes apparent, the vessels in this case usually invade a perfectly clear cornea.

### SUMMARY

Young rats, from 26 to 78 gm. in weight, which were given a tryptophane-deficient diet developed cataractous changes in from 8 to 82 days. These changes were manifested with or without a concurrent lysine deficiency. The lenticular changes were prevented by supplementation of the diets with 0.1% or 0.2% tryptophane, either when the diet was restricted or given ad libitum. A large proportion of the animals which received diets deficient in both tryptophane and lysine or lacking in either of these amino acids developed vascularity of the cornea. A few showed a generalized ophthalmia.

With the aid of the ophthalmoscope the cataract seen in these animals could be readily distinguished from that resulting both from riboflavin deficiency and from the feeding of galactose or xylose. The vascularity of the cornea seen in these amino acid-deficient animals showed certain features which distinguished it from the vascularity of riboflavin deficiency.

In addition to the ocular lesions noted, other changes associated with the tryptophane deficiency were loss of weight, hunchback, unkempt appearance, alopecia, greasy hair, and nervousness.

## LITERATURE CITED

BERG, C. P., AND MARTHA POTGIETER 1932 Tryptophane metabolism. II. The growth-promoting ability of *dl*-tryptophane. J. Biol. Chem., vol. 94, p. 661.

BERG, C. P., AND W. C. ROSE 1929 Tryptophane and growth. J. Biol. Chem., vol. 82, p. 479.

CURTIS, P. B., S. M. HAUGE AND H. R. KRAYBILL 1932 The nutritive value of certain animal protein concentrates. J. Nutrition, vol. 5, p. 503.

DARBY, W. J., AND PAUL L. DAY 1938 The riboflavin content of meats. J. Nutrition, vol. 16, p. 209.

———— 1939 Xylose as a cataractogenic agent. Proc. Soc. Exp. Biol. and Med., vol. 41, p. 507.

DAY, PAUL L., W. J. DARBY AND W. C. LANGSTON 1937 The identity of flavin with the cataract-preventive vitamin. J. Nutrition, vol. 13, p. 389.

DAY, PAUL L., W. C. LANGSTON AND C. S. O'BRIEN 1931 Cataract and other ocular changes in vitamin G deficiency. Am. J. Ophth., vol. 14, p. 1005.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, p. 273.

MITCHELL, H. S. 1935 Cataract in rats fed on galactose. Proc. Soc. Exp. Biol. and Med., vol. 32, p. 971.

MITCHELL, H. S., AND W. M. DODGE 1934 Nutritional cataract in rats. J. Biol. Chem., vol. 105, p. lxi (Proc.).

TOTTER, J. R., AND PAUL L. DAY 1941 Cataract and other ocular changes resulting from tryptophane deficiency. J. Biol. Chem., vol. 140, p. cxxxiv (Proc.).

# NICOTINIC ACID, PANTOTHENIC ACID AND PYRIDOXINE IN WHEAT AND WHEAT PRODUCTS [1]

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Since wheat is an important constituent of the average American diet, it is obviously desirable to extend our knowledge about the vitamin content of this cereal. The flour enrichment program has increased the interest in this problem. Booth ('40) has reported on the thiamine content of wheats grown in various parts of the world. Schultz, Atkin and Frey ('39, '41) have made a survey of the vitamin $B_1$ content of American cereals. Nordgren and Andrews ('41) have studied the vitamin $B_1$ content of American and Canadian wheats, while Downs and Cathcart ('41) have made a study of the thiamine content of commercial wheats of the 1940 crop. Andrews, Boyd and Terry ('42) and Conner and Straub ('41 a, '41 b) have recently studied the distribution of riboflavin in cereals. Taylor ('41), Tobey and Cathcart ('41) and Tisdall et al. ('41) have discussed problems concerned with the fortification program, and have presented data on the distribution of thiamine and riboflavin in wheat and wheat products. Bin-

nington and Andrews ('41) have recently reported on the distribution of vitamin E in products of cereal milling.

As far as we know, no systematic study of the distribution of other vitamins in wheat has been reported. In our investigation, a number of varieties of wheat grown in various parts of the United States and Canada have been assayed for their nicotinic acid, pantothenic acid, and pyridoxine content.

<center>EXPERIMENTAL</center>

The nicotinic acid assays were made by the microbiological method of Snell and Wright ('41). This method employs the response of Lactobacillus arabinosus 17-5 to nicotinic acid or nicotinamide. The response is measured by titrating the acid produced. Samples were prepared for analysis as follows: 0.2 gm. of finely-ground wheat was suspended in 75 cc. of $H_2O$, 20 cc. of 8% NaOH were added, and the suspension was autoclaved for 20 minutes at 15 pounds pressure. It was then neutralized with HCl and diluted to 200 cc. Treatment of the sample on the steam bath for 1 hour with 5 N HCl gave identical results. Recovery of added nicotinic acid ranged from 90% to 110%.

It has been found by various workers that the microbiological assay of a water suspension of wheat for nicotinic acid gives values approximately 20% lower than the assay of a NaOH treated suspension. The effect is apparently not one of increased extraction, since a water filtrate is similarly increased in potency on treatment with NaOH. It would seem that the increase is due to the freeing of nicotinic acid from an inactive compound. This inactive compound is extremely labile to alkali since ¼% NaOH at room temperature gives the full effect within 5 minutes. Treatment with moderately strong acid or prolonged heating at neutrality will also bring about the conversion. Studies are now in progress to determine whether the increased activity, as measured by the microorganisms (after alkali treatment), is due to a compound biologically active in the animal body.

Pantothenic acid was determined by the microbiological method of Strong, Feeney and Earle ('41). In this method the response of Lactobacillus casei ε to pantothenic acid is measured by titrating the acid produced. Preparation of the sample was carried out as follows: 0.2 gm. of finely-ground wheat was suspended in 100 cc. of $H_2O$, autoclaved for 20 minutes at 15 pounds pressure, and diluted to 500 cc. Recovery of added pantothenic acid ranged from 90% to 110%. Strong, Feeney and Earle have found that treatment of cereals with enzymes does not produce a significant change in apparent pantothenic acid as measured by the microbiological method. Values are expressed in micrograms of calcium pantothenate per gram.

Pyridoxine was determined by the biological method of Conger and Elvehjem ('41). Twenty-one-day-old rats were depleted for 2 weeks on a pyridoxine low ration and the ration was then supplemented with the sample. Crystalline pyridoxine hydrochloride was fed at levels of 50, 75 and 100 µg. per 100 gm. of ration. The pyridoxine content of the sample was determined by interpolation on the standard curve obtained; values are given in micrograms of vitamin $B_6 \cdot HCl$ per gram.

In order to guard against group variation due to errors in assay procedure, representative samples from the various groups were assayed at the same time. All values are expressed as micrograms per gram air dry sample.

### RESULTS AND DISCUSSION

Table 1 gives the results of analyses of four varieties of dark hard winter wheat grown at four different localities in Kansas and Nebraska. The Chiefkan variety samples from all locations and each of the varieties grown at Hutchinson contain slightly more nicotinic acid than the other samples. In the case of pantothenic acid and pyridoxine, neither varietal nor environmental differences seem to have any significant effect on the amount present. However, the Blackhull variety

TABLE 1

*Nicotinic acid, pantothenic acid and pyridoxine content of dark hard winter wheats.*

| VARIETY | LOCATION GROWN | | | | |
|---|---|---|---|---|---|
| | LINCOLN | DODGE CITY | WICHITA | HUTCHINSON | AVERAGE |
| | $\mu g./gm.$ | $\mu g./gm.$ | $\mu g./gm.$ | $\mu g./gm.$ | $\mu g./gm.$ |
| | | | Nicotinic acid | | |
| Chiefkan | 61 | 67 | 66 | 66 | 66 |
| Blackhull | 57 | 60 | 53 | 59 | 59 |
| Turkey | 53 | 54 | 61 | 58 | 58 |
| Tenmarq | 56 | 54 | 56 | 57 | 57 |
| Average | 57 | 59 | 59 | 60 | 60 |
| | | | Pantothenic acid | | |
| Chiefkan | 13.7 | 14.9 | 15.3 | 14.4 | 14.6 |
| Blackhull | 13.1 | 15.3 | 13.1 | 15.8 | 14.3 |
| Turkey | 14.1 | 15.6 | 16.0 | 15.5 | 15.3 |
| Tenmarq | 15.2 | 15.4 | 15.5 | 17.5 | 15.9 |
| Average | 14.0 | 15.3 | 15.2 | 15.8 | 15.0 |
| | | | Pyridoxine | | |
| Chiefkan | 4.4 | 4.1 | 5.2 | 4.3 | 4.5 |
| Blackhull | 4.0 | 4.2 | 3.7 | 3.5 | 3.8 |
| Turkey | 5.4 | 4.6 | 5.8 | 5.7 | 5.4 |
| Tenmarq | 5.4 | 6.1 | 4.1 | 4.4 | 5.0 |
| Average | 4.8 | 4.8 | 4.7 | 4.5 | 4.7 |

samples contain appreciably lower amounts of pyridoxine than the other varieties.

In table 2 the values for miscellaneous dark hard winter wheats grown in Kansas, Minnesota, Oklahoma, Texas and Washington are given. The range of concentration of the three vitamins in these samples is approximately the same as in the samples obtained from Kansas and Nebraska listed in table 1, but the values tend to be lower. None of the dark hard winter wheats listed in table 2 contains as much nicotinic acid as the average of the samples reported in table 1. There is considerable overlapping in the case of pantothenic acid and pyridoxine in this group, but the average values in table 2 are appreciably lower than those in table 1 as may be seen from the data given in table 3. Table 2 also gives the values

TABLE 2

*Nicotinic acid, pantothenic acid and pyridoxine content of miscellaneous wheats.*

| VARIETY | SOURCE | NICOTINIC ACID | PANTO-THENIC ACID | PYRI-DOXINE |
|---|---|---|---|---|
| | | $\mu g./gm.$ | $\mu g./gm.$ | $\mu g./gm.$ |
| *Dark Hard Winter Wheats:* | | | | |
| Turkey | Manhattan, Kansas | 50 | 14.4 | 3.7 |
| Turkey | Meno, Oklahoma | 55 | 16.0 | 4.1 |
| Turkey | Pullman, Washington | 51 | 9.1 | 3.2 |
| Tenmarq | Orienta, Oklahoma | 52 | 12.4 | 3.5 |
| Chiefkan | Guthrie, Oklahoma | 51 | 11.8 | 4.7 |
| Blackhull | Eagle City, Oklahoma | 50 | 13.1 | 4.1 |
| Blackhull | Claude, Texas | 53 | 12.6 | 5.6 |
| Minturki | Minneapolis, Minnesota | 51 | 14.5 | 3.2 |
| Montana | Minneapolis, Minnesota | 47 | 11.9 | 3.7 |
| Kharkof | Pullman, Washington | 58 | 12.7 | 5.4 |
| *Soft Red Winter Wheats:* | | | | |
| Kawvale | Manhattan, Kansas | 52 | 14.4 | 4.6 |
| Kawvale | Lincoln, Nebraska | 59 | 15.7 | 6.3 |
| Kawvale | Talala, Oklahoma | 55 | 15.1 | 3.9 |
| Fulcaster | Wooster, Ohio | 58 | 10.4 | 4.2 |
| Wabash | Wooster, Ohio | 52 | 9.3 | 4.9 |
| Red Rock | Wooster, Ohio | 62 | 9.4 | 4.7 |
| Fultz | Wooster, Ohio | 67 | 10.6 | 4.8 |
| Thorne | Wooster, Ohio | 58 | 9.4 | 4.6 |
| Purdue | Wooster, Ohio | 61 | 9.4 | 4.6 |
| *Soft White Winter Wheats:* | | | | |
| American Banner | Wooster, Ohio | 62 | 11.4 | 4.7 |
| Yorkwin | Wooster, Ohio | 59 | 10.9 | 5.0 |
| Dawson | Wooster, Ohio | 65 | 10.9 | 4.3 |
| *White Club Wheat:* | | | | |
| Hymar | Pullman, Washington | 56 | 11.4 | 4.5 |
| *Dark Northern Spring Wheats:* | | | | |
| Ceres | Minneapolis, Minnesota | 52 | 15.0 | 3.6 |
| Marquis | Minneapolis, Minnesota | 48 | 15.3 | 3.8 |
| Thatcher | Minneapolis, Minnesota | 67 | 17.0 | 4.2 |
| Nordhaugen | Minneapolis, Minnesota | 53 | 13.9 | 4.0 |
| Red Bobs | Winnepeg, Manitoba | 76 | 14.7 | 5.6 |
| Thatcher | Winnepeg, Manitoba | 106 | 13.3 | 5.1 |
| Garnet | Winnepeg, Manitoba | 59 | 11.0 | 5.1 |
| Reward | Winnepeg, Manitoba | 64 | 12.5 | 5.1 |
| Renown | Winnepeg, Manitoba | 70 | 14.7 | 4.8 |
| Garnet | LaCombe, Alta. | 55 | 9.8 | 3.8 |
| Thatcher | Saltcoats, Sask. | 77 | 14.0 | 4.6 |
| *Hard White Spring Wheats:* | | | | |
| Baart No. 38 | Pullman, Washington | 53 | 11.3 | 4.8 |
| Federation | Pullman, Washington | 57 | 11.4 | 4.4 |
| Blue Stem | Pullman, Washington | 56 | 15.2 | 4.7 |
| Burbank | Minneapolis, Minnesota | 53 | 15.0 | 5.5 |
| *Soft White Spring Wheat:* | | | | |
| Dicklow | Ogden, Utah | 56 | 11.9 | 4.9 |

obtained on miscellaneous soft winter wheats. The nicotinic acid and pyridoxine contents of these samples fall in the regular range of the other samples studied, while the pantothenic acid content of all the wheats obtained from Wooster, Ohio, is comparatively low (table 3). These results indicate that genetic differences are of little consequence in determining the pantothenic acid content of soft winter wheat, while environmental differences have considerable effect. The values obtained on miscellaneous spring wheats are also shown in

TABLE 3

*Range of values for contents of nicotinic acid, pantothenic acid and pyridoxine.*

| SOURCE | NUMBER OF SAMPLES | NICOTINIC ACID | | PANTOTHENIC ACID | | PYRIDOXINE | |
|---|---|---|---|---|---|---|---|
| | | Ave. | Range | Ave | Range | Ave. | Range |
| | | $\mu g./gm$ | | $\mu g./gm$ | | $\mu g./gm$ | |
| Kansas and Nebraska | 16 | 60 | 53–71 | 15.0 | 13.1–17.5 | 4.7 | 3.5–6.1 |
| Kansas, Minnesota, Oklahoma, Texas and Washington | 10 | 52 | 47–58 | 12.8 | 9.1–16.0 | 4.1 | 3.2–5.6 |
| Wooster, Ohio | 9 | | | 10.2 | 9.3–11.4 | | |
| Kansas, Nebraska, Oklahoma and Washington | 4 | | | 14.1 | 11.1–16.7 | | |
| Canada | 7 | 74 | 55–106 | | | | |
| Minnesota, Utah and Washington | 9 | 53 | 48–67 | | | | |

table 2. Samples obtained from Canada are definitely higher in nicotinic acid content (table 3). No corresponding variation was found in the case of pantothenic acid and pyridoxine. The hard white spring wheats from Minnesota and Washington listed in table 2 show a vitamin content that falls in the range of the other samples studied.

More than fifty samples of commercially milled patent and clear flours have been assayed for nicotinic acid. Three samples giving typical values have also been assayed for pantothenic acid and pyridoxine. The results, which are collected in table 4, indicate that patent flour, compared with whole wheat, contains approximately one-sixth as much nicotinic acid, and

about half as much pantothenic acid and pyridoxine. It has been shown that in the milling of patent flour, thiamine is left behind in the coarser fractions in proportions comparable to the nicotinic acid. If one wishes to restore the vitamin balance of whole wheat to patent flour, it is of major importance to add thiamine and nicotinic acid.

It is interesting to note that although there is a concentration of thiamine, riboflavin, and pyridoxine in the germ of the wheat kernel, wheat germ is about equal to whole wheat in pantothenic acid and tends to be lower than whole wheat in nicotinic acid. The use of small amounts of wheat germ in flour, which some have advocated as a measure to give flour

TABLE 4

*Nicotinic acid, pantothenic acid and pyridoxine in wheat flours and wheat germ.*

| SAMPLE | NICOTINIC ACID | LOSS IN MILLING | PANTO-THENIC ACID | LOSS IN MILLING | PYRI-DOXINE | LOSS IN MILLING |
|---|---|---|---|---|---|---|
| | $\mu g./gm$ | % | $\mu g./gm.$ | % | $\mu g /gm.$ | % |
| Average value for 55 samples of whole wheat | 59 | | 13.3 | | 4.6 | |
| Patent flour | 10 | 83 | 5.7 | 57 | 2.2 | 52 |
| First clear flour | 21 | 64 | 9.6 | 28 | 3.9 | 15 |
| Second clear flour | 57 | 3 | 12.8 | 4 | 5.7 | |
| Wheat germ | 34 | | 15.3 | | 9.6 | |

more nearly the nutritive properties of whole wheat, means a contribution of much less pantothenic acid and nicotinic acid than vitamin $B_1$, riboflavin, or vitamin $B_6$. It would appear that the great improvement which is imparted by using 5% of germ in bread (such as reported by McHenry, '40) cannot be attributed to the addition of these two members of the B-complex.

## SUMMARY

Fifty-five samples of wheat, varying as to variety and source, have been assayed for nicotinic acid, pantothenic acid, and pyridoxine. Patent flour, clear flours, and wheat germ have also been assayed for these vitamins. Patent flour, compared

with whole wheat, was found to contain about one-sixth as much nicotinic acid, and approximately one-half as much pantothenic acid and pyridoxine.

## LITERATURE CITED

ANDREWS, J. S., H. M. BOYD AND D. E. TERRY 1942 The riboflavin content of cereal grains and bread and its distribution in products of wheat milling. Cereal Chem., vol. 19, p. 55.

BINNINGTON, D. S., AND J. S. ANDREWS 1941 The distribution of vitamin E in products of cereal milling. Cereal Chem., vol. 18, p. 678.

BOOTH, R. G. 1940 The thiochrome method for the estimation of aneurin, with a survey of the aneurin content of wheats. J. Soc. Chem. Ind., vol. 59, p. 181.

CONGER, T. W., AND C. A. ELVEHJEM 1941 The biological estimation of pyridoxine (vitamin B₆). J. Biol. Chem., vol. 138, p. 555.

CONNER, R. T., AND G. J. STRAUB 1941 a The determination of thiamin by the thiochrome reaction. Ind. and Eng. Chem., vol. 13, p. 380.

———— 1941 b The combined determination of riboflavin and thiamin in food products. Ind. and Eng. Chem., vol. 13, p. 385.

DOWNS, D. E., AND W. H. CATHCART 1941 The thiamin content of commercial wheats of the 1940 crop. Cereal Chem., vol. 18, p. 796.

McHENRY, E. W. 1940 Observations on the nutritive value of bread. Can. Public Health J., vol. 31, p. 428.

NORDGREN, R., AND J. S. ANDREWS 1941 The thiamin content of cereal grains. Cereal Chem., vol. 18, p. 802.

SCHULTZ, A. S., L. ATKIN AND C. S. FREY 1941 A preliminary survey of the vitamin B₁ content of American cereals. Cereal Chem., vol. 18, p. 106.

———— 1939 The vitamin B₁ content of wheat, flour and bread. Cereal Chem., vol. 16, p. 643.

SNELL, E. E., AND L. D. WRIGHT 1941 A microbiological method for the determination of nicotinic acid. J. Biol. Chem., vol. 139, p. 625.

STRONG, F. M., R. E. FEENEY AND A. EARLE 1941 Microbiological assay for pantothenic acid. Ind. and Eng. Chem., vol. 13, p. 566.

TAYLOR, A. E. 1941 Why enrichment of flour? Wheat Studies of the Food Res. Inst., vol. 18, p. 77.

TISDALL, F. F., S. H. JACKSON, T. G. H. DRAKE, L. H. NEWMAN, A. G. O. WHITESIDE, H. MILLER AND J. EDGAR 1941 The retention of the wheat vitamins in flour and bread — a problem of national importance. The Canadian Med. Assoc. J., vol. 45, p. 101.

TOBEY, J. A., AND W. H. CATHCART 1941 Fortification and restoration in the baking and dairy industries. Ind. and Eng. Chem., vol. 33, p. 714.

# THE RELATION OF B-VITAMINS AND DIETARY FAT TO THE LIPOTROPIC ACTION OF CHOLINE [1]

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Junkersdorf and Kohl ('26) observed that choline lowered liver-fat levels in dogs. A similar choline effect was demonstrated in rats by Best, Hershey and Huntsman ('32), and by a number of workers since that time. Studies concerning the relation of the B-vitamins to liver-fat metabolism originated when Whipple and Church ('36) demonstrated that thiamine increases liver-fat in the rat. These results were confirmed by McHenry ('37). Gavin and McHenry ('40) reported that the thiamine-induced fatty liver was not affected by pyridoxine, nicotinic acid, or riboflavin, but that choline administration restored liver-fat to normal levels. Engel and Phillips ('39) observed increases in liver-fat in thiamine-deficient rats which were fed thiamine, but they were unable to prevent this condition with choline. This lack of agreement suggests that other B-vitamins might be concerned with the metabolism of liver-fat, since the diet employed by the latter workers contained a B-vitamin concentrate (liver-filtrate factor).

McHenry and Gavin ('40) found that a beef liver fraction containing pantothenic acid and "factor W" produced a marked increase in liver-fat which could be prevented by "lipocaic," a pancreatic extract. More recently Gavin and McHenry ('41) have shown that a similar fatty liver could be produced by biotin and prevented by inositol.

Halliday ('38) reported that rats on pyridoxine-deficient diets had fatty livers which could not be entirely prevented by choline. Gavin and McHenry ('40) were unable to confirm these results.

The above citations strongly indicate that a number of B-vitamins are concerned with the metabolism of liver fat. In most of the work cited, these vitamin effects were produced in animals receiving diets which were high in fats or low in casein. Casein is known to have a lipotropic action (Channon and Wilkinson, '35; Beeston et al., '35). It seemed desirable, therefore, to determine whether liver-fat metabolism could be influenced by the addition of accessory food factors to a diet containing 18% of casein. Such diets are in common use in nutrition studies. Furthermore, it was of interest to investigate the lipotropic action of choline under such dietary conditions and to determine if normal levels of liver-fat could be maintained in growing rats receiving a purified diet supplemented with crystalline vitamins.

<center>MATERIALS AND METHODS</center>

The basal diet used in these studies consisted of casein [2] 18, sucrose 78, and salt mixture [3] 4%. The diet was supplemented once weekly with adequate amounts of carotene and calciferol. The remaining accessory food factors were supplied daily, mixed with a small portion of the basal diet. As a routine procedure, litters of eight rats were divided into two like groups as to number, weight, and sex. By such means it was possible to study the effect of a given vitamin within a litter.

The rats were weaned at 23 days of age, placed in individual cages with raised screen floors, and, unless otherwise indicated, were fed the experimental diet for a period of 3 weeks. At the end of this period the animals were killed by carotid bleeding. The livers were weighed, dried at 104°C., reweighed, ground, and extracted with dry ethyl ether for 16 hours. The liver-fat values reported represent the per cent ether extract

[2] Labco, from Borden Company.
[3] J. Biol. Chem., vol. 89, p. 199, 1930.

on a dry weight basis. In order to determine normal values for liver-fat in rats of this age, livers from twenty-four animals which had been fed the stock diet for 3 weeks were treated in the manner described above. Data thus obtained showed that the average liver-fat in such animals was 5.25 ± 0.20%.

## RESULTS

*The relation of thiamine, riboflavin, pyridoxine, and pantothenic acid to the lipotropic action of choline.* The relation of thiamine, riboflavin, pyridoxine, and pantothenic acid to the metabolism of choline was studied by omitting them singly from the diet and comparing the data thus obtained with data obtained when all these factors were present in the diet. The various levels at which choline was fed, and the daily supplements of the other accessory factors are given in the accompanying tables. The results of this series of experiments are presented in table 1.

When thiamine, riboflavin, or pantothenic acid was omitted from the diet, approximately normal values for liver-fat were obtained even though the daily choline chloride intake was only 2 mg. The need of additional dietary choline as a lipotropic agent was apparent when thiamine, riboflavin, and pantothenic acid were all present in the diet. The increased need for choline was correlated with increased food consumption and with increased body weight. The omission of pyridoxine from the diet for a 3-week experimental period had no effect on liver-fat values.

That the abnormal liver-fat deposition in the rats receiving both low levels of choline and all the B-vitamins was mainly caused by a lack of dietary choline is demonstrated by the reduction in liver-fat which occurred with increased choline feeding. At the 2 mg. level of choline chloride, hemorrhagic kidneys were frequently encountered, and in a few instances death occurred from choline deficiency. When the choline chloride intake was raised from 2 mg. to 5 mg. per rat daily, no hemorrhagic kidneys were observed. This increase in dietary choline had a marked lipotropic effect and also pro-

## TABLE 1

*Average growth, food consumption, and percentage liver fat under varying B-vitamin and choline intake.*

| GROUP [1] | DAILY CHOLINE CHLORIDE INTAKE | THIAMINE | | | | RIBOFLAVIN | | | | PANTOTHENIC ACID | | | | PYRIDOXINE | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Body weight [2] | | Daily food intake | Liver fat | Body weight [2] | | Daily food intake | Liver fat | Body weight [2] | | Daily food intake | Liver fat | Body weight [2] | | Daily food intake | Liver fat |
| | mg. | gm. | | gm. | % | gm. | | gm. | % | gm. | | gm. | % | gm. | | gm. | % |
| A | 2 | 39 | 71 | … | 33.10 | 38 | 62 | … | 38.50 | 45 | 75 | … | 26.85 | 40 | 72 | … | 28.85 |
| B | 2 | 42 | 33 | … | 5.10 | 37 | 41 | … | 9.25 | 42 | 56 | … | 9.90 | 44 | 59 | … | 21.95 |
| A | 5 | 45 | 90 | 6.1 | 18.50 | 42 | 84 | 5.0 | 12.80 | 47 | 90 | 5.8 | 17.90 | 42 | 78 | 5.4 | 18.60 |
| B | 5 | 46 | 50 | 3.2 | 6.30 | 41 | 52 | 3.0 | 7.65 | 47 | 79 | 5.0 | 14.30 | 43 | 65 | 4.1 | 18.20 |
| A | 10 | 54 | 112 | 7.7 | 12.40 | 42 | 88 | 6.0 | 10.15 | 39 | 82 | … | 12.60 | 42 | 88 | 6.0 | 11.75 |
| B | 10 | 53 | 53 | 3.0 | 5.00 | 44 | 48 | 3.1 | 3.90 | 40 | 78 | … | 6.00 | 42 | 66 | 4.1 | 11.15 |
| A | 20 | 56 | 108 | 6.2 | 10.15 | 55 | 96 | 5.0 | 10.20 | 50 | 88 | 6.1 | 7.95 | 55 | 96 | 5.1 | 7.15 |
| B | 20 | 55 | 60 | 3.4 | 5.60 | 54 | 60 | 3.6 | 3.60 | 48 | 78 | 5.4 | 6.00 | 54 | 82 | 3.8 | 6.40 |
| A | 40 | 57 | 106 | … | 10.70 | 45 | 75 | 4.7 | 8.70 | 57 | 79 | … | 7.00 | 43 | 91 | … | 13.85 |
| B | 40 | 55 | 50 | … | 5.05 | 44 | 49 | 3.0 | 7.85 | 55 | 75 | … | 4.90 | 42 | 64 | … | 10.15 |
| A | 70 | 49 | 87 | 5.2 | 11.20 | 48 | 79 | 6.3 | 9.05 | 44 | 78 | 4.7 | 7.40 | 39 | 87 | 4.5 | 7.60 |
| B | 70 | 49 | 44 | 2.8 | 5.20 | 48 | 55 | 3.5 | 7.45 | 46 | 76 | 4.2 | 4.25 | 40 | 65 | 3.5 | 11.65 |

[1] Each rat in group A received daily 20 µg. of thiamine, riboflavin, and pyridoxine; 100 µg. of calcium pantothenate, and 0.1 ml. of corn oil. The animals in group B received the same supplements with B-vitamins omitted, one at a time, as indicated by the table headings. Each experimental result represents an average obtained from eight rats.

[2] The first column of figures represents initial body weight, the second column represents final body weight.

duced a definite growth response. Although the results were somewhat variable, in general it would appear that, under these dietary conditions, 10 mg. of choline chloride per rat daily produced the maximum lipotropic effect. Feeding amounts larger than 10 mg. failed to lower the liver-fat values to any significant degree, and there was some indication that the higher levels (40 to 70 mg.) resulted in a slight growth inhibition.

Dietary choline, even at high levels, failed to restore liver-fat values to normal if the diet contained thiamine, riboflavin, calcium pantothenate, and pyridoxine. The absence of other lipotropic agents from the diet seemed a logical explanation. Since inositol has been demonstrated to have a lipotropic action under certain dietary conditions (Gavin et al., '41) the action of this substance was determined in the following series of experiments.

*The relation of inositol to the lipotropic action of choline.* To determine the action of inositol, rats were fed the basal diet plus thiamine, riboflavin, pyridoxine, pantothenic acid and inositol as the B-vitamin supplements. Litter-mate rats receiving the same diet and supplements, with the exception of inositol, served as controls. Choline was again fed to both groups at varying levels. The amounts of the various supplements fed are presented with the results in table 2.

As in the previous experiments, the animals receiving thiamine, riboflavin, pyridoxine, pantothenic acid, and adequate choline had liver-fat values appreciably above normal. With the addition of inositol, however, the liver-fat was reduced to the levels found in rats receiving a stock diet. The results show clearly that both choline and inositol are necessary as lipotropic agents for rats receiving purified diets containing thiamine, riboflavin, pyridoxine, and pantothenic acid.

*The relation of pyridoxine to the lipotropic action of choline as influenced by the duration of the experiment.* In the previous experiments it was apparent that pyridoxine had no effect on liver-fat levels if the test feeding period was limited to

TABLE 2

*Average growth, food consumption, and percentage liver fat with and without inositol in the diet, and with varying levels of choline intake.*

| GROUP [1] | DAILY CHOLINE CHLORIDE INTAKE | BODY WEIGHT | | DAILY FOOD INTAKE | LIVER FAT |
|---|---|---|---|---|---|
| | | Initial | Final | | |
| | *mg.* | *gm.* | *gm.* | *gm.* | *%* |
| A | 5 | 46 | 92 | 6.0 | 20.25 |
| B | 5 | 46 | 94 | 6.2 | 14.10 |
| A | 10 | 47 | 95 | 6.0 | 8.80 |
| B | 10 | 48 | 96 | 6.0 | 6.35 |
| A | 20 | 44 | 88 | 5.8 | 8.70 |
| B | 20 | 45 | 88 | 5.6 | 5.75 |
| A | 40 | 48 | 92 | 4.9 | 6.50 |
| B | 40 | 48 | 90 | 4.8 | 5.10 |

[1] Each rat in group A received daily, 20 μg. each of thiamine, riboflavin, and pyridoxine, 100 μg. of calcium pantothenate, and 0.1 ml. of corn oil. Those in group B received the same supplement plus 3 mg. of inositol per rat daily. Each experimental result represents an average obtained from twelve rats.

3 weeks. Since it has been reported (Halliday, '38) that rats deficient in pyridoxine have fatty livers, it seemed desirable to determine the influence of this factor on the lipotropic action of choline under more severe deficiency conditions. In these experiments each rat received 10 mg. of choline chloride daily. The vitamin supplements which the rats received daily over varying experimental periods are presented with the results in table 3.

After a 3-week feeding period the pyridoxine-deficient animals had liver-fat values comparable to those of the control group receiving pyridoxine. Similar results were obtained after a 6-week period. By the end of the eighth week, however, the pyridoxine-deficient animals had accumulated abnormal quantities of liver fat. These abnormal liver-fat levels were also present in the rats receiving the pyridoxine-deficient diet for 14 and 24 weeks, respectively.

On the basis of these results it would seem that choline was less effective as a lipotropic agent in rats receiving a diet deficient in pyridoxine than in rats receiving the same diet

supplemented with pyridoxine. The results indicate that the animals receiving pyridoxine in this series of experiments had liver-fat values somewhat higher than those reported for rats receiving the stock diet. On the basis of the results with inositol in the preceding experiments it would seem that this abnormality was due to a deficiency of inositol. Experiments are in progress to clarify this point.

TABLE 3

*The effects on liver-fat levels of essential fatty acids (corn oil) and pyridoxine in the diet.*

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **DIET WITH AND WITHOUT PYRIDOXINE** | | | | | **DIET WITH AND WITHOUT ESSENTIAL FATTY ACIDS (CORN OIL)** | | | | |
| Group [1] | Period | Average body weight | | Average liver fat | Group [2] | Period | Average body weight | | Average liver fat |
| | | Initial | Final | | | | Initial | Final | |
| | *weeks* | *gm* | *gm* | *%* | | *weeks* | *gm* | *gm.* | *%* |
| A | 3 | 41 | 82 | 11.2 | A | 3 | 42 | 89 | 11.4 |
| B | 3 | 42 | 96 | 11.8 | B | 3 | 41 | 94 | 11.7 |
| A | 6 | 45 | 85 | 12.6 | A | 8 | 44 | 120 | 25.0 |
| B | 6 | 43 | 135 | 13.2 | B | 8 | 46 | 166 | 11.5 |
| A | 8 | 45 | 93 | 22.5 | A | 10 | 48 | 124 | 25.8 |
| B | 8 | 43 | 143 | 11.4 | B | 10 | 50 | 199 | 13.5 |
| A | 14 | 44 | 85 | 16.8 | A | 12 | 49 | 168 | 18.3 |
| B | 14 | 45 | 190 | 9.5 | B | 12 | 48 | 244 | 8.9 |
| A | 24 | 44 | 86 | 38.5 | A | 20 | 44 | 197 | 20.8 |
| B | 24 | 45 | 221 | 17.6 | B | 20 | 44 | 290 | 15.2 |

[1] Each rat in group A received daily 20 µg. each of thiamine and riboflavin, 100 µg. of calcium pantothenate, 0.1 ml. of corn oil, and 10 mg. of choline chloride. Those of group B received the same supplement plus 20 µg. of pyridoxine per rat daily. Each experimental result represents an average obtained from four rats.

[2] Each rat in group A received daily 10 µg. each of thiamine and pyridoxine, 0.5 gm. of Lilly's liver extract 343, and 10 mg. of choline chloride. Those in group B received the same supplement plus 0.1 ml. of corn oil (Mazola) per rat daily. Each experimental result represents an average obtained from four rats.

*The relation of essential fatty acids (corn oil) to the lipotropic action of choline as influenced by the duration of the experiment.* In the previous experiments corn oil was included in the diet as a source of essential fatty acids. Preliminary trials had indicated that this dietary supplement had no in-

fluence on the lipotropic action of choline over a 3-week experimental period. Since other investigators have reported that essential fatty acids and pyridoxine mutually function in curing rat acrodynia, and since pyridoxine influenced the lipotropic action of choline in experiments of longer duration, it seemed desirable to conduct a series of experiments with, and without, corn oil in the diet. For these experiments the casein of the basal diet was subjected to four 2-hour extractions with boiling 95% ethyl alcohol. The accessory food factors fed daily are shown with the results in table 3. Choline chloride was again fed at a level of 10 mg. per rat daily.

The liver-fat levels in the rats receiving corn oil showed no significant changes regardless of the duration of the experiment. After an 8-week feeding period the liver-fat in the rats receiving the fat-free diet had increased approximately 100%. Similar increases were observed if the experimental period was extended to 10, 12, or 20 weeks.

These results indicate that the 10 mg. daily choline intake was less effective as a lipotropic agent in the fat-deficient animal than it was in the animals receiving a source of essential fatty acids. Thus it would seem that pyridoxine and essential fatty acids act mutually in influencing the lipotropic action of choline and are complementary in this respect.

### DISCUSSION

The basal diet used in these studies is comparable to those used by many investigators concerned with the nutrition of the various B-vitamins. The results reported herein indicate that the abnormal liver-fat deposition resulting from the feeding of thiamine, riboflavin, and pantothenic acid should not be considered as a harmful effect of these vitamins. This abnormality was entirely alleviated by fortifying the diet with adequate amounts of the known lipotropic agents, choline and inositol, and must be regarded therefore as representing a deficiency of these factors. Both choline and inositol are

essential, in a purified diet containing 18% casein and supplemented with crystalline vitamins, if normal levels of liver-fat are to be maintained.

It is difficult to compare the choline requirement as found in the present study with those reported by other investigators, because of variations in the diets. Griffith ('41) has reported that, for diets containing 18 to 24% of casein and 6% of yeast, 4 to 6 mg. of choline chloride was needed daily to prevent the accumulation of liver lipids. Data herein presented indicate that at least 10 mg. of choline chloride per rat daily is necessary for the maintenance of normal liver-fat levels. Work now in progress on chemical and biological assays of various yeasts for their choline content indicates that the choline requirement suggested by Griffith is in good agreement with that found in the present investigation, if the choline supplied by the yeast is taken into account.

That pyridoxine and corn oil decrease the deposition of liver fat is of interest in view of the reports that both these factors are concerned in the cure of rat acrodynia (Birch, '38; Salmon, '38, '40). The observation that fatty livers result if rats are deprived of pyridoxine over long periods of time is a confirmation of results reported by Halliday ('38). Gavin and McHenry ('40) failed to observe any effect of pyridoxine on liver-fat if rats were fed first a depletion diet for 3 weeks and then various test supplements including pyridoxine for 1 week. Present results indicate that an 8-week depletion period is necessary before a pyridoxine effect can be demonstrated.

It is of interest to note that inositol and choline are both lipotropic agents and that both have been reported to have a protective action against perosis in chicks. Wiese and associates ('38) reported that inositol afforded protection against perosis in chicks receiving insufficient manganese. Hegsted and associates ('41) and Hogan and associates ('41) have reported that choline prevents perosis.

## SUMMARY AND CONCLUSIONS

When thiamine, riboflavin, pantothenic acid, pyridoxine, corn oil, and choline were fed to rats receiving a purified diet containing 18% of casein for a 3-week experimental period, an abnormal accumulation of liver-fat resulted. Under these conditions 2 mg. of choline chloride per rat daily failed to prevent the kidney hemorrhages of choline deficiency; at least 10 mg. of choline chloride was necessary for this factor to exert its maximum lipotropic action but normal liver-fat levels were still not obtained.

The addition of 3 mg. of inositol per rat daily to the diet adequate in choline and containing the above B-vitamins reduced the liver fat to the normal level found in rats receiving an adequate stock diet.

Prolonged feeding of a diet deficient in pyridoxine or essential fatty acids resulted in fatty livers, even though the diet contained adequate choline.

It is concluded that pyridoxine and a source of essential fatty acids are necessary in the diet for choline·to function properly as a lipotropic agent. Inositol, in addition to choline, is a necessary dietary constituent for the rat receiving purified diets supplemented with the other B-vitamins known to be required by this species.

### LITERATURE CITED

BEESTON, A. W., H. J. CHANNON AND H. WILKINSON 1935 The influence of the cascinogen content of diets on the nature of the ''cholesterol'' fatty liver. Biochem. J., vol. 29, p. 2659.

BEST, C. H., J. M. HERSHEY AND M. E. HUNTSMAN 1932 The control of the deposition of liver fat. Am. J. Physiol., vol. 101, p. 7.

BIRCH, T. W. 1938 The relation between vitamin B and the unsaturated fatty acid factor. J. Biol. Chem., vol. 124, p. 775.

CHANNON, H. J., AND H. WILKINSON 1935 Protein and the dietary production of fatty livers. Biochem. J., vol. 29, p. 350.

ENGEL, R. W., AND P. H. PHILLIPS 1939 Fatty livers as a result of thiamin administration in vitamin B₁ deficiency of the rat and the chick. J. Nutrition, vol. 18, p. 329.

GAVIN, G., AND E. W. McHENRY 1940 The B-vitamins and fat metabolism. III. The effects of vitamin B₆ upon liver and body fat. J. Biol. Chem., vol. 132, p. 41.

———— 1941 Inositol: a lipotropic factor. J. Biol. Chem., vol. 139, p. 485.

GRIFFITH, W. H. 1941 The nutritional importance of choline. Editorial Review. J. Nutrition, vol. 22, p. 239.

HALLIDAY, N. 1938 Fatty livers in vitamin B₆ deficient rats. J. Nutrition, vol. 16, p. 285.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., vol. 138, p. 459.

HOGAN, A. G., L. R. RICHARDSON, H. PATRICK AND H. L. KEMPSTER 1941 Perosis due to a vitamin deficiency. J. Nutrition, vol. 21, p. 327.

JUNKERSDORF, P., AND A. KOHL 1926 Effect of definite hormones on metabolic physiology. II. Choline during hunger. Arch. Ges. Physiol. (Pflügers), vol. 211, p. 612.

McHENRY, E. W. 1937 Vitamin B₁ and fatty livers. J. Physiol., vol. 89, p. 287.

McHENRY, E. W., AND G. GAVIN 1940 Effects of a beef liver fraction on fat synthesis in rats. Science, vol. 91, p. 171.

SALMON, W. D. 1938 The effect of certain oils in alleviating localized erythematous dermatitis (acrodynia or vitamin B₆ deficiency) in rats. J. Biol. Chem., Proc. Soc. Biol. Chem., vol. 123, p. civ.

———— 1940 The supplementary relationship of vitamin B₆ and unsaturated fatty acids. J. Biol. Chem., Proc. Soc. Biol. Chem., vol. 133, p. lxxxiii.

WHIPPLE, D. V., AND C. F. CHURCH 1936 The composition of growth induced by vitamins B (B₁). J. Biol. Chem., Proc. Soc. Biol. Chem., vol. 109, p. cvii.

WIESE, A. C., B. C. JOHNSON, C. A. ELVEHJEM AND E. B. HART 1938 Phosphorus metabolism of chicks afflicted with perosis. Science, vol. 88, p. 383.

# FACTORS REQUIRED BY CHICKS MAINTAINED ON A HEATED DIET [1]

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In 1932 a heated grain and casein diet was first used in our laboratory for nutritional studies with chicks (Kline, Keenan, Elvehjem and Hart, '32). This ration was found to be useful in studies dealing with the identification of pantothenic acid as the chick antidermatitis factor (Woolley, Waisman and Elvehjem, '39) and for the assay of pantothenic acid in food materials (Waisman, Mickelsen and Elvehjem, '39). While this ration was quite satisfactory for such studies, it soon became apparent that factors other than pantothenic acid were necessary to render the diet complete. We wish to report here the results of several years of work on the heated diet and the data that have been obtained on further factors required by the growing chick.

## EXPERIMENTAL

The basal diet used throughout was a modification of the original diet described by Kline, Keenan, Elvehjem and Hart ('32). It consists (in per cent) of ground yellow corn 56, standard wheat middlings 25, crude casein 12, soy bean oil 3, and a salt mixture 4. The chicks were given 2 drops halibut liver oil weekly to insure the supply of vitamins A and D. Although the original workers resorted to 100-hour

heating of the grains and casein part of the diet at 100°C., later work (Mickelsen, Waisman and Elvehjem, '38) showed that a 30-hour treatment of the diet at 120°C. gave more consistent dermatitis.

In the majority of our experiments 100 gm. of the basal ration were supplemented with 300 µg. thiamine, 400 µg. riboflavin, 400 µg. pyridoxine, 100 mg. choline, and 0.50 mg. of 2-methyl,-1,4-naphthoquinone. There was no growth stimulation when all of these vitamins were included in the basal ration, but the chicks did show greater survival time.

### Pantothenic acid requirement

The availability of crystalline calcium pantothenate enabled us to study both the level necessary to completely protect the birds from the typical dermatitis and the level required to give maximum growth. A large number of groups of day-old White Leghorn chicks were fed the basal diet (with the vitamins mentioned above) plus levels of calcium pantothenate ranging from 100 µg. to 2000 µg. per 100 gm. of diet. Typical weights of the birds are given in table 1. Complete protection from all symptoms was obtained with 300 µg. of calcium pantothenate per 100 gm. of diet. Below this figure there were cases of the typical incrustations.

There was observed a corresponding growth increase with each increment of the vitamin up to 1000 µg. per 100 gm. of diet. In several groups a 750 µg. level gave nearly maximum growth and only one or two times did the average weight of the chicks on the 1000 µg. level exceed the growth obtained on the 750 µg. level. The variation is thought to be due to storage of other essential factors in different batches of chicks. The maximum growth obtained on 750 µg. is in contrast to that reported by Jukes ('39) who found 1400 µg. to give optimum growth on a similar ration. It should be pointed out, however, that Jukes depleted the chicks for a short period before the pantothenic acid was given, while our chicks received the pantothenate from the start. The data do show that the

amount of vitamin needed for prevention of the dermatitis is less than that necessary to promote maximum growth.

It was apparent from these experiments that the chicks receiving the heated diet plus adequate amounts of calcium pantothenate did not give the growth considered to be optimum for White Leghorn chicks. The optimum weight for this breed in our laboratory has been found to be about 230 gm. at 4 weeks of age. In an effort to attain optimum growth in the chicks fed the heated diet, experiments were devised to study the effect of several food factors which had been proposed for the chick, but which had not been chemically defined. To this

TABLE 1

*Typical growth responses of chicks maintained on the heated diet to increasing levels of calcium pantothenate.*

|  | WEIGHT OF BIRDS AT 4 WEEKS | PANTOTHENIC ACID DERMATITIS |
|---|---|---|
|  | *gm.* |  |
| 241H basal | 65 | ++++ |
| + 100 μg. P.A.[1]/100 gm. | 85 | +++ |
| + 200 μg. P.A./100 gm. | 98 | ++ |
| + 300 μg. P.A./100 gm. | 111 | — |
| + 500 μg. P.A./100 gm. | 122 | — |
| + 750 μg. P.A./100 gm. | 132 | — |
| + 1000 μg. P.A./100 gm. | 148 | — |
| + 15 mg. P.A./kg. | 133 | — |

[1] Calcium pantothenate.

end a variety of liver fractions, yeast preparations and other sources of vitamins were added to the basal ration plus adequate amounts of calcium pantothenate. Because of the large amount of data gathered during our extended experiments, the material will be presented in highly condensed form with a short discussion of the individual fractions concerned. Typical responses to a supplement in a given series are presented so that the weight gain can be compared to that for the control group of that series. Each weight listed in the table is the average of at least six chicks in a group. Each supplement was tested in at least three different series of experiments. The pertinent data are summarized in table 2.

TABLE 2

*Typical growth responses by chicks fed various supplements together with the heated diet.*[1]

| SUPPLEMENTS TO 100 GM. OF RATION 241 H. | AVERAGE WT. IN 4 WEEKS | SUPPLEMENTS TO 100 GM. OF RATION 241 H. | AVERAGE WT. IN 4 WEEKS |
|---|---|---|---|
| *Factor U* | | *Para-aminobenzoic acid and vitamin K* | |
| 1.5 mg. P.A.[2] | 102 | 241H + 1.5 mg. P.A. + | |
| Factor U + 1.5 mg. P.A. | 128 | 40 mg. PABA | 133 [4] |
| | | 1241H [6] basal | 37 [3] |
| *Cartilage* | | 1241H + 5 mg. vitamin K | 39 [3] |
| 20% cartilage | 66 [3] | 1241H + 1.5 mg. P.A. + | |
| 1.5 mg. P.A. | 101 | 5 mg. vitamin K | 47 |
| 1.5 mg. P.A. + 20% cartilage | 100 [4] | 1241H + 15 mg. P.A. + | |
| 1.5 mg. P.A. + factor U | 124 [4] | 40 mg. PABA + 5 mg. | |
| 1.5 mg. P.A. + factor U + | | vitamin K | 52 |
| 10% cartilage | 163 [4] | *Inositol* | |
| 1.5 mg. P.A. + 3% | | 1.5 mg. P.A. | 132 |
| S.L.E.[5] +10% cartilage | 164 [4] | 1.5 mg. P.A. + 100 mg. | |
| 1.5 mg. P.A. + 3% S.L.E. + | | inositol | 137 |
| 0.5% cystine + 0.5% | | 1.5 mg. P.A. + 40 mg. PABA | |
| arginine + 3% glycine | 152 [4] | +100 mg. inositol | 151 |
| | | *All factors* | |
| *Biotin* | | 1.5 mg. P.A. + 3% S.L.E. | |
| 5 or 10% molasses | 79 [3] | +10% molasses + 10% | |
| 1.5 mg. P.A. | 124 | cartilage | 170 |
| 1.5 mg. P.A. + 10% molasses | 133 | 1.5 mg. P.A. + 10% molasses | |
| 1.5 mg. P.A. + factor U + | | +100 mg. inositol + 10% | |
| 10% molasses | 169 | cartilage | 168 [4] |
| 5% liver residue | 87 | 1.5 mg. P.A. + 3% S.L.E. | |
| 5% L.R. + 1.5 mg. P.A. | 177 | + 100 mg. inositol + | |
| 5% heated L.R. + 1.5 mg. | | 10% cartilage | 165 [4] |
| P.A. | 125 | 1.5 mg. P.A. + 10% molasses | |
| 1.5 mg. P.A. + 3% S.L.E. | 164 | +10% cartilage + 100 mg. | |
| + 10% cartilage | (210)[4] | inositol + 3% S.L.E. | 193 |
| 1.5 mg. P.A. + 3% S.L.E. + | 213 | 1.5 mg. P.A. + 10% molasses | |
| 10% cartilage + 5% L.R. | (280) | + 3% S.L.E. + 100 mg. | |
| 1.5 mg. P.A. + 3% S.L.E. + | | inositol | 154 [4] |
| 10% cartilage + 15 μg. | 205 | 1.5 mg. P.A. + 3% S.L.E. | |
| biotin conc. | (273) | + 10% cartilage + 15 μg. | |
| | | biotin | 210 |

[1] Figures within parentheses give average weights of the chicks when 5 weeks of age.

[2] Calcium pantothenate.

[3] Pantothenic acid deficiency symptoms.

[4] Biotin deficiency symptoms.

[5] Solubilized liver extract, a source of factor U.

[6] One hundred-hour dry heating of ration (120°C.).

## Factor U

Stokstad and Manning ('38) first attributed growth responses in chicks to a methyl alcohol-water soluble fraction of yeast. This soluble fraction which was then treated with fuller's earth and the eluate obtained by treatment with mild alkali, was called "Factor U." When this eluate fraction was added to our heated diet at a level equivalent to 10% yeast a definite growth response over that obtained with adequate quantities of calcium pantothenate alone resulted. In our more recent trials a "solubilized liver extract" was used as the source of the factors in the factor U fraction of yeast. At 4 weeks of age the chicks receiving calcium pantothenate plus this preparation were on the average 25 to 30 gm. heavier than those on the basal diet. The effect of the added factor U was observed in several instances where it was added (1) with calcium pantothenate alone, (2) with calcium pantothenate and molasses, or (3) with calcium pantothenate and cartilage. The factor U preparation was found to have little effect on the growth of chicks fed the basal diet with no added calcium pantothenate. It is evident that calcium pantothenate must be included before any study can be made of additional factors required by chicks fed this diet.

## Cartilage growth factor

The necessity of supplying the "cartilage growth factor" to purified rations for chicks in order to obtain increased growth has been pointed out by Hegsted et al. (Hegsted, Hier, Elvehjem and Hart, '41). It has been shown (Almquist, Stokstad, Mecchi and Manning, '40; Almquist and Mecchi, '40) that chicks require glycine and certain carbohydrates for normal growth, and Hegsted, Briggs, Elvehjem and Hart ('41) have shown that arginine produces marked responses in chicks fed simplified diets. Arginine and glycine are able to replace cartilage to a large extent when fed together, and are able to give better feathering as well as improved growth. It was previously shown by Arnold et al. ('36) that the arginine

requirement of the chick is greater than can be supplied by 18 parts of casein. Although the addition of 10% casein did not improve the growth of our chicks maintained on the heated diet, it did appear advisable to feed cartilage both as a source of certain amino acids and for other factors which exist in this preparation. When either 10 or 20% cartilage was added to the basal diet there was no growth response whatever, and the addition of either 10 or 20% cartilage to the ration containing adequate calcium pantothenate also failed to produce a response when compared with that obtained on the ration containing calcium pantothenate alone. The marked response obtained when 10% cartilage is added to the ration supplemented with calcium pantothenate and a factor U concentrate contrasts sharply with the growth obtained with pantothenic acid and factor U alone. It appears then that the limiting deficiency in the heated diet in addition to pantothenic acid is one or more of the factors contained in the factor U preparation. Only after this deficiency is satisfied can the growth effect due to cartilage be obtained. On the basis of chemical properties and the method of preparation of the factor U fraction, it is unlikely that the cartilage factors are contained in the yeast fraction. The addition of 1% arginine, 1% cystine and 3% glycine gave somewhat less growth than 10% cartilage when the factor U was supplied by the solubilized liver residue.

### Biotin

Throughout several years of work on this ration we had observed in chicks receiving the basal heated diet marked incrustations at the corners of the mouth, and scaliness and fissuring on the bottom of the feet and in between the toes. When adequate calcium pantothenate was included in the diet, the incrustations at the corners of the beak were absent while the dry scaly callousness on the bottom of the feet persisted. This scaly dermatitis was similar to that described by Hegsted et al. ('40).

We had observed in several series of experiments that both liver residue and molasses were able to prevent the leg

dermatitis. Subsequent work conclusively demonstrated to us that the active factor in these materials was biotin. When liver residue, which is the insoluble material remaining after the hot water extraction of fresh liver, was fed at a 5% level plus calcium pantothenate, there was obtained a definite response in growth together with the complete prevention of all dermatitis. The liver residue was undoubtedly able to supply sufficient factor U and biotin to account for the growth obtained. When 10% molasses was fed, enough biotin was present for the prevention of the leg dermatitis but growth was limited by the absence of factor U.

When the liver residue was heated for 30 hours at 120°C. in a manner similar to the heat treatment of the basal ration, a decrease in growth resulted, as can be seen from table 2. In most cases heating the liver residue reduced the growth response by nearly one-third, and this was an indication that it contained a heat labile factor which was destroyed by dry heat treatment. It would appear that this heat labile factor is related to one of the factors in the factor U preparation since good growth was again obtained if heated liver residue was fed together with solubilized liver extract.

Several groups of chicks were fed biotin [2] at levels of 15 µg. per 100 gm. of the ration with different combinations of the supplements. No scaly dermatitis was observed in the groups receiving the biotin and again growth was very much improved. The report of Ansbacher and Landy ('41) cited the fact that as little as 1.25 µg. of biotin per day by either oral, subcutaneous or intramuscular administration was able to cure the dermatosis in their chicks maintained on a diet similar to ours but heated for a much longer period. Our chicks received at least 1.5 µg. per day if it is granted that the birds ate an average of 10 gm. of feed a day. An important difference between the work of Ansbacher and Landy and this report is that our diet is more complete and thus accounts for the better growth of our chicks. It is quite possible therefore that the increased weight of our birds demanded a greater

[2] Obtained from SMA Corp., Chagrin Falls, Ohio.

quantity of biotin than was supplied by 10% molasses. Patrick et al. ('41) found that biotin prevented the dermatitis in turkey poults under their conditions rather than riboflavin as reported by Jukes ('38). The importance of considering the detailed procedure and conditions of each investigator in explaining the results obtained cannot be overemphasized. Hegsted et al. ('42) have fixed the biotin requirement of the chick at 7–10 µg. per 100 gm. of a purified diet. We have made no attempt to establish the biotin requirement of chicks fed the heated diet.

### Para-aminobenzoic acid, vitamin K and inositol

It has been reported by Ansbacher ('41 a, '41 b) that both vitamin K and para-aminobenzoic acid (PABA) produce growth responses on a 168-hour heated diet. All our attempts to confirm this work by using a 100-hour heated (120°C.) basal diet in which vitamin K was omitted have met with failure. Levels of 40 mg. PABA per 100 gm. of diet did not give any growth response over that obtained with adequate levels of calcium pantothenate. Addition of vitamin K in the form of 2-methyl,-1,4-naphthoquinone was also ineffective at the levels fed. When both vitamin K and PABA were added to the diet no increase in the weight of the chicks was observed beyond that due to pantothenic acid alone.

The addition of PABA to the 30-hour heated diet was also ineffective in increasing the growth obtained beyond that due to additions of calcium pantothenate alone. The growth response with pantothenic acid on the excessively heated diet of Ansbacher was also less than on the ordinary heated diet and this may be ascribed to destruction of several additional factors by the prolonged heat treatment. We must conclude that under our conditions no response was obtained in chicks fed either vitamin K or PABA. It would seem appropriate to retest the effect of PABA and vitamin K on the heated diet when the latter is supplemented with those limiting factors described in this report.

Hegsted, Briggs, Mills, Elvehjem and Hart ('41) have presented some preliminary data which indicate that the growing

chick requires inositol for normal nutrition. Inositol was fed as a supplement to the heated diet at a 0.1% level both alone and together with other factors. Experiments with twenty or more groups of chicks have presented data which would indicate that under our conditions, using the heated ration, there is no growth effect with inositol, probably due to the fact that the phytin in the grains of our diet is not destroyed by the heat treatment.

<div align="center">DISCUSSION</div>

The data obtained from our experiments clearly indicate that the amount of calcium pantothenate required for complete prevention of pantothenic acid deficiency dermatitis in chicks is approximately one-half that necessary for maximum growth on the heated diet. The data also show that a factor or group of factors contained in the factor U concentrate from yeast or liver is required by growing chicks fed the heated diet. The addition of cartilage was without effect when added alone, but in conjunction with factor U supplements there was a marked increase in growth. The added growth due to factor U and to cartilage was only obtained when the primary deficiency, namely, lack of pantothenic acid, was completely prevented. Repeated trials have conclusively proved that addition of individual growth factor concentrates to the basal ration gave growth responses which were directly correlated to the pantothenic acid content of the supplements.

The data indicate that added biotin is required for growth by chicks maintained on the heated diet. The dermatitis which develops primarily on the legs is usually seen in those birds which have received a ration supplemented with various concentrates and which have been maintained on the diet beyond 4 weeks. Good growth is evidently necessary in order to produce the symptoms in the chick. The growth responses due to molasses and liver residue were at first believed to be due to several substances in these materials, but later our experiments definitely indicated that biotin alone could account for the increased growth obtained. That biotin was concerned

in the growth response and in the prevention of scaly dermatitis by liver residue was further demonstrated in the complete lack of leg dermatitis in those chicks receiving this supplement.

The dry heating of the grain and casein ration evidently destroys the biotin as well as the pantothenic acid and factor U present in these natural materials; growth is better and no dermatitis is observed in chicks fed the unheated diet. It was of interest therefore to note that heating the liver residue in a manner similar to that of the basal diet caused a greater incidence of dermatitis together with a decrease in the growth response. The decrease due to the heating seemed to be less marked when the diet was improved by the addition of several growth factor concentrates. The greatest differences were observed when only calcium pantothenate was included with the heated and unheated liver residue.

It is our impression from the data here presented that liver residue contains both biotin and factor U. Certain preliminary evidence (Hutchings et al., '41) has indicated that a chick eluate factor (factor U) and a bacterial growth factor (since named "folic acid" by Mitchell et al. ('41)) are identical. It is presumed by us that in addition to biotin the other heat labile factor in liver residue may be identical with "folic acid" or one of the factors in the factor U fraction.

Under the conditions of our experiments we have not been able to substantiate the claims of Ansbacher ('41 a) that para-aminobenzoic acid is required by chicks maintained on the heated diet. The data would also indicate that inositol was without any activity when added to the heated diet.

Nearly optimum growth was obtained when the heated ration was supplemented with biotin, cartilage, factor U and pantothenic acid. When any of the four supplements was omitted the growth did not compare favorably with that obtained when all the fractions were supplied. The addition of 5% of yeast or 3% of whole liver did not produce better growth than the combination of the fractions.

### SUMMARY

In addition to the six crystalline factors of the B group of vitamins, growing chicks maintained on the heated diet require the eluate factor in yeast (factor U), cartilage, and biotin. Factor U and biotin may be supplied by liver residue. Pantothenic acid, biotin and one or more factors in the factor U fraction are destroyed by the dry heat treatment.

### LITERATURE CITED

ALMQUIST, H. J., E. L. R. STOKSTAD, E. MECCHI AND P. D. V. MANNING 1940 Identification of the rice factor. J. Biol. Chem., vol. 134, p. 213.

ALMQUIST, H. J., AND E. MECCHI 1940 Identification of the rice factor. The essential nature of the glycine component. J. Biol. Chem., vol. 135, p. 355.

ANSBACHER, S. 1941 a p-Aminobenzoic acid, a vitamin. Science, vol. 93, p. 164.

———— 1941 b Additional observations on vitamin K deficient diets. Proc. Soc. Exp. Biol. and Med., vol. 46, p. 421.

ANSBACHER, S., AND M. LANDY 1941 Biotin and scaly dermatosis in the chick. Proc. Soc. Exp. Biol. and Med., vol. 48, p. 3.

ARNOLD, A., O. L. KLINE, C. A. ELVEHJEM AND E. B. HART 1936 Further studies on the growth factor required by chicks. The essential nature of arginine. J. Biol. Chem., vol. 116, p. 699.

HEGSTED, D. M., J. J. OLFSON, R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1940 Studies on a dermatitis in chicks distinct from pantothenic acid deficiency. J. Nutrition, vol. 20, p. 599.

HEGSTED, D. M., G. M. BRIGGS, R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Inositol in chick nutrition. Proc. Soc. Exp. Biol. and Med., vol. 47, p. 376.

HEGSTED, D. M., S. W. HIER, C. A. ELVEHJEM AND E. B. HART 1941 Growth factors in cartilage for the chick. J. Biol. Chem., vol. 139, p. 863.

HEGSTED, D. M., G. M. BRIGGS, C. A. ELVEHJEM AND E. B. HART 1941 Role of arginine and glycine in chick nutrition. J. Biol. and Chem., vol. 140, p. 191.

HEGSTED, D. M., R. C. MILLS, G. M. BRIGGS, C. A. ELVEHJEM AND E. B. HART 1942 Biotin in chick nutrition. J. Nutrition, vol. 23, p. 175.

HUTCHINGS, B. C., N. BOHONOS, D. M. HEGSTED, C. A. ELVEHJEM AND W. H. PETERSON 1941 Relation of a growth factor required by lactobacillus casei e to the nutrition of the chick. J. Biol. Chem., vol. 140, p. 681.

JUKES, T. H. 1938 The vitamin G requirements of young poults. Poultry Science, vol. 17, p. 227.

———— 1939 Pantothenic acid requirement of the chick. J. Biol. Chem., vol. 129, p. 225.

KLINE, O. L., J. A. KEENAN, C. A. ELVEHJEM AND E. B. HART 1932 Use of the chick in vitamin $B_1$ and $B_2$ studies. J. Biol. Chem., vol. 99, p. 295.

MICKELSEN, O., H. A. WAISMAN AND C. A. ELVEHJEM 1938 Inactivity of nicotinic acid in chick dermatitis. J. Biol. Chem., vol. 124, p. 313.

MITCHELL, H. K., E. E. SNELL AND R. J. WILLIAMS 1941 The concentration of "folic acid." J. Am. Chem. Soc., vol. 63, p. 2284.

PATRICK, H., R. V. BOUCHER, R. A. DUTCHER AND H. C. KNADEL 1941 Biotin and prevention of dermatitis in turkey poults. Proc. Soc. Exp. Biol. and Med., vol. 48, p. 456.

STOKSTAD, E. L. R., AND P. D. V. MANNING 1938 Evidence of a new growth factor required by chicks. J. Biol. Chem., vol. 125, p. 687.

WAISMAN, H. A., O. MICKELSEN AND C. A. ELVEHJEM 1939 Distribution of the chick antidermatitis factor (pantothenic acid) in meats and meat products. J. Nutrition, vol. 18, p. 247.

WOOLLEY, D. W., H. A. WAISMAN AND C. A. ELVEHJEM 1939 Studies on the structure of the chick antidermatitis factor. J. Biol. Chem., vol. 192, p. 673.

# THE ABSORPTION AND RETENTION OF CAROTENE AND VITAMIN A BY HENS ON NORMAL AND LOW FAT RATIONS [1]

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ONE FIGURE

The efficiency of absorption and utilization of carotene and of vitamin A under various conditions has been the subject of several investigations. Ahmad ('31) found that carotene from either raw carrots or cooked spinach was absorbed nearly twice as well by humans when the diet contained fat as when the fat was omitted. That fat aids in the absorption of carotene was also found by van Eekelen and Pannevis ('38). Basu ('37) claimed that fat is essential for the absorption by rats of both carotene and vitamin A. On the other hand, De ('37) and Wilson, Das Gupta and Ahmad ('37); working with rats and humans, respectively, found no difference between the absorption of vitamin A on diets containing high and low levels of fat. Gray, Hickman and Brown ('40) reported that rats stored in the liver nearly six times as much vitamin A from cod liver oil as from β-carotene. Very little work has been done using the hen as an experimental animal.

Record, Bethke and Wilder ('37) and Peterson, Hughes and Payne ('39) found no difference between the absorption of carotene and of vitamin A by hens fed normal diets. A recent

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communication from this laboratory (Russell, Taylor and Polskin, '40) reported that chicks fed a diet containing less than 0.1% fat were able to utilize sufficient crystalline carotene to maintain normal growth for 14 weeks. However, the carotene was fed at a level approximately five times the minimum requirement, since these studies were concerned mainly with fat requirements. In the present work attention is given primarily to the effect of normal and low fat diets on the absorption and retention of carotene and vitamin A.

### EXPERIMENTAL

White Leghorn hens were housed in individual pens, with wire mesh bottoms, in a fattening battery and fed an all-mash ration of the following composition: white corn meal 20, wheat flour middlings 20, wheat bran 20, pulverized whole oats 20, meat scrap (55% protein) 10, skimmed milk powder 5, ether-extracted alfalfa leaf meal 5, calcite flour 2, and sodium chloride 1. This constituted the normal ration. The alfalfa was extracted with ether in order to reduce as much as possible the carotene content of the ration. The low fat ration was prepared by extracting the normal ration in percolators at room temperature with a mixture of 3 parts diethyl ether and 1 part 95% ethyl alcohol. As determined by 16 hours extraction with diethyl ether in a Soxhlet extractor, the fat content was 0.07%. Sucrose was added to the low fat diet at a 4% level to replace the fat which had been removed. Two per cent of yeast [2] washed with several changes of ether, was added to both rations to ensure an adequate supply of the vitamin B complex. All birds received 10 minutes of ultraviolet radiation from a quartz-mercury vapor lamp three times a week, the distance from the burner to the floor of the cage being about 60 cm.

The carotene and vitamin A supplements were fed daily by capsule. The factors were dissolved in diethyl ether, an appropriate amount pipetted into capsules, and the solvent evaporated. The beginning and end of each period was identified

[2] Northwestern Yeast Powder.

by feeding carmine or charcoal and the last vitamin feeding was given at least 20 hours before the end of a period. The excreta were collected daily, put into 95% ethyl alcohol, and stored in a refrigerator (40°C.) until analyses could be made. Tests showed that the factors were stable under these conditions. Carotene was determined in the food and in the droppings by the method of Peterson, Hughes and Freeman ('37), the final solutions being read with a Fisher Electrophotometer using a Corning no. 430 filter. Vitamin A was determined by the Carr-Price reaction ('26), the readings being made at 615 mμ with a Bausch and Lomb Universal Spectrophotometer.

### FEEDING STUDIES WITH CAROTENE

White Leghorn hens were maintained without added carotene on the normal and low fat rations for a depletion period of 10 weeks, during which time feed consumption was measured and droppings collected in periods 1, 2, and 3, and for the normal ration group in period 4, as shown in table 1. In period 4, which began at the end of the depletion period, a quantity of crystalline carotene, calculated to be equal to that consumed in the normal ration, was fed to the birds on the low-fat ration. In periods 5 and 6, which followed immediately, increased amounts of carotene were fed to both groups. Attempts were made in periods 4, 5, and 6, to equalize the carotene intake of the two groups.

### Results and discussion

In period 1 (table 1) it was noted that the total yellow pigment excreted by hens on the low fat ration, expressed as carotene, was greater than the quantity of carotene consumed. To determine the nature of the pigment in the extract, absorption curves were obtained by means of a Bausch and Lomb Universal Spectrophotometer and compared with curves for both purified carotene and an extract of excreta from the normal group. The results showed that the predominant pigment in the excreta of the low fat group was not carotene (fig. 1), whereas the extract from the normal group showed

a curve characteristic of this substance. Since the pigment was predominantly not carotene, and it was assumed that hens on the normal ration would excrete at least as much of the unknown pigment as those of the low fat group, it seemed advisable to apply at least a minimum correction to the values obtained with the normal ration. The quantities deducted in

TABLE 1

*Daily absorption of carotene by the hen on normal and low fat rations.*

| PERIOD NO.[1] | NO. OF HENS | DAYS ON EXPT | FOOD CONSUMP-TION | CAROTENE CONSUMED | | | TOTAL YELLOW PIGMENT EXCRETED EXPRESSED AS CAROTENE | CARO-TENE EX-CRETED | CAROTENE ABSORBED | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | In feed | By capsule | Total | | | | |
| | | | *gm.* | *μg.* | *μg.* | *μg.* | *μg.* | *μg.* | *μg.* | *%* |
| 1 N[2] | 4 | 0 | 58.8 | 60 | 0 | 60 | 38 | 26 | 34 | 57 |
| LF | 12 | | 78.4 | 8 | 0 | 8 | 12 | 0 | 8 | 100 |
| 2 N | 2 | 11 | 44.1 | 45 | 0 | 45 | 20 | 15 | 30 | 67 |
| LF | 2 | | 49.0 | 5 | 0 | 5 | 5 | 0 | 5 | 100 |
| 3 N | 8 | 43 | 62.7 | 64 | 0 | 64 | 32 | 28 | 36 | 56 |
| LF | 8 | | 58.8 | 6 | 0 | 6 | 4 | 0 | 6 | 100 |
| 4 N | 4 | 71 | 75.5 | 77 | 0 | 77 | 44 | 40 | 37 | 48 |
| LF | 4 | | 78.4 | 8 | 58 | 66 | 47 | 43 | 23 | 35 |
| 5 N | 4 | 74 | 72.6 | 74 | 67 | 141 | 58 | 54 | 87 | 61 |
| LF | 4 | | 78.4 | 8 | 117 | 125 | 99 | 95 | 30 | 24 |
| 6 N | 4 | 79 | 69.6 | 71 | 200 | 271 | 92 | 88 | 183 | 67 |
| LF | 4 | | 78.4 | 8 | 232 | 240 | 205 | 201 | 39 | 16 |

[1] Periods 1, 4, 5 and 6 were each 3 days and periods 2 and 3 were 4 days in length.

[2] N — Normal ration: 3.83% fat; 102 μg. carotene per 100 gm.; LF — Low fat ration: 0.07% fat; 10 μg. carotene per 100 gm.

periods 1, 2, and 3 were those observed on the low fat ration during the corresponding periods. It was assumed that in period 3, excretion of the non-carotene pigment had reached a minimum and therefore this value, 4 μg., was subtracted in periods 4, 5, and 6. Kemmerer and Fraps ('38) reported the presence of a non-carotenoid pigment of unknown character in the excrement of rats and chickens receiving feeds containing negligible amounts of carotene. Presumably the pigment found in the present investigation was the same as

that reported by Kemmerer and Fraps but no attempt was made to determine its nature.

The absorption of carotene on the normal ration at the beginning of the experiment was 57% and for period 4, after 71 days on the ration, 48%. Except for period 2, in which observations were made with only two hens, the percentage absorption tended to decrease as the experiment progressed. In the case of hens on the low fat feed, carotene was not excreted during the first 3 periods as shown by spectrophoto-
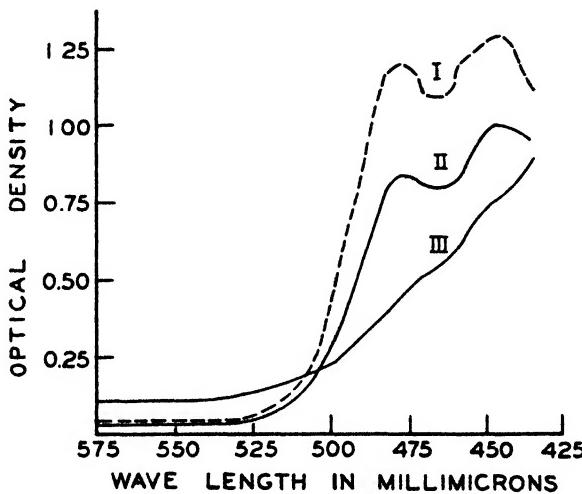


Fig. 1 Absorption curves for purified carotene (I), for the extract from excreta of birds receiving carotene (II), and for the yellow coloring matter from excreta of birds fed practically no carotene (III).

metric data and therefore it was concluded that the small quantity of carotene in the feed, assuming no destruction in the tract, was absorbed.

When the carotene intake was increased by feeding crystalline carotene by capsule so that essentially the same quantity was consumed by each group, the hens on the normal ration showed an increasing tendency to absorb this factor. The quantity of carotene absorbed was definitely less on the low fat ration than on the normal. Although the quantity absorbed increased with increasing dosage, there was a pro-

gressive decrease in the percentage absorbed. Thus, when the intake on the low fat ration was increased 3.5 times, the quantity absorbed increased only 1.7 times while under the same conditions on the normal ration, the increase was five-fold. Since the fat content of the ration is the differentiating factor, it is concluded that fat is necessary in the poultry ration for satisfactory absorption of carotene. The quantity of carotene absorbed may be limited by the quantity of fat present and it is possible that in period 6, when 67% of the carotene fed in the normal ration was absorbed, the birds may have been approaching maximum absorption. On the other hand, Kemmerer and Fraps ('38) reported that as the quantity of carotene supplied in the form of dehydrated alfalfa leaf meal was increased in the ration, the percentage absorption decreased. The difference between the results of the two investigations is probably due to the availability of carotene for absorption, the crystalline form, in the presence of fat, being more readily available.

### INJECTION STUDIES WITH CAROTENE

The question was raised as to whether any of the absorbed carotene appeared in the excreta. In an attempt to answer this question, injection studies were conducted parallel with the feeding studies of periods 4, 5, and 6. The birds used were from the same group as in the previous studies and had the same preliminary period on the normal and low fat diets. A carotene suspension in water made according to the methods described by Fodor and Schoenfeld ('31) was injected daily, intravenously, in four birds in both the normal and low fat groups. An attempt was made to inject essentially the same quantity of carotene as was fed daily by capsule during periods 4, 5, and 6, although this was not possible in every case. Also, each group was given approximately the same level of carotene according to the average feed consumption as determined during the depletion period. The ration, experimental procedure, and analytical methods were the same as those in the preceding part of this investigation.

## Results and discussion

As will be seen in table 2, when as much as 195 μg. of carotene was injected intravenously, daily, in the low fat birds, none was excreted. The carotene excreted by the hens on the normal ration for periods 8 and 9, based on the amount consumed in the feed but not on the additional amount which was injected, was 32% and 36%, respectively. These values are

TABLE 2

*Daily retention of carotene injected intravenously in hens on normal and low fat rations.*

| PERIOD NO.[1] | DAYS ON EXPT. | FOOD CONSUMP- TION | CAROTENE ADMINISTERED | | | YELLOW PIGMENT EXCRETED | CAROTENE EXCRETED | |
|---|---|---|---|---|---|---|---|---|
| | | | In feed | Injected | Total | | | |
| | | *gm.* | *μg* | *μg* | *μg* | *μg* | *μg* | %[2] |
| 7 N[3] | 71 | 53.9 | 55 | 0 | 55 | 27 | 22 | 40 |
| LF | 71 | 68.6 | 7 | 49 | 56 | 5 | 0 | 0 |
| 8 N | 74 | 46.1 | 47 | 53 | 100 | 20 | 15 | 32 |
| LF | 74 | 58.8 | 6 | 98 | 104 | 5 | 0 | 0 |
| 9 N | 79 | 56.9 | 58 | 180 | 238 | 27 | 21 | 36 |
| LF | 79 | 68.6 | 7 | 195 | 202 | 6 | 0 | 0 |

[1] The collection periods were 3 days in length. Each value is an average of the results from 4 hens.
[2] Calculated on the basis of the carotene in the feed.
[3] N — Normal ration: 3.83% fat. LF — Low fat ration: 0.07% fat.

lower than those obtained in periods 1 to 4 inclusive which, by calculation from the absorption values in table 1, ranged from 33% to 52%. In period 9-N the excretion of the pigment was 36% of that in the feed, despite the daily injection of 180 μg. of carotene. It is believed therefore that the carotene which appears in the excreta is that which has escaped absorption and not that excreted into the tract.

### VITAMIN A STUDIES

## Experimental

Studies of the absorption and retention of vitamin A by hens fed normal and low fat diets were carried out in much the same manner as in the case of carotene. The birds under-

went a preliminary depletion period which varied from 2
to 24 weeks, the variation in time being due to the addition
of new birds to an older group which had been used in a
previous experiment. Vitamin A ester was fed by capsule
in increasing amounts during 13 successive 3-day periods and
the quantity which appeared in the droppings was determined.
The ester was a molecular distilled concentrate having a
potency of 200,000 U.S.P. units per gram and was dissolved
in ether for the filling of capsules.

For 1 month after the last feeding of the vitamin the hens
were continued on the two rations. At the end of this period
they were sacrificed and the vitamin A content of the livers
determined.

### Results and discussion

During the first 7 periods (table 3) vitamin A was increased
progressively from 375 units in period 2, to 8250 units in
period 7, a total of 18,850 units being fed during these periods.
Despite this large intake of vitamin A, only two birds in the
normal and one in the low fat group showed what was in-
terpreted to be traces of the factor in the droppings. When
an additional 12,500 units was fed in period 8, making a total
to the end of that period of 31,350 units, the excreta of two
of the normal and one of the low fat birds contained the factor.
After an additional 18,300 units was fed in period 9, making
a grand total of 49,650 units, vitamin A appeared definitely
in the excreta of all members of the two groups except one,
no. 1043. A small amount of vitamin A appeared in the excreta
of this hen when an additional 24,800 units was fed in period
10. It is of interest to note that throughout the experiment
this individual excreted the lowest percentage of vitamin A
intake of any of the birds.

The fact that vitamin A was not detected during the first
7 periods, does not necessarily mean that none was excreted.
If it is assumed that the hens failed to absorb the same per-
centage of the vitamin during these periods as later in the
experiment, the calculated amounts which would have been

present in the droppings of these early periods should have been very small. These amounts could not be measured by the technique employed, partly because of a small error inherent in reading the spectrophotometer but more because of the fleeting nature of the color developed and the presence of a relatively high level of non-vitamin A chromogens which produced a somewhat variable general absorption.

TABLE 3

*Excretion of vitamin A by hens on normal and low fat rations — 3-day periods.*

| PERIOD NO. | VITAMIN A ESTER FED DURING A 3-DAY PERIOD U.S.P. XI UNITS | PER CENT OF VITAMIN A EXCRETED | | | | | | |
| | | NORMAL GROUP | | | | LOW FAT GROUP | | |
| | | Bird No. 1022 | Bird No. 1316 | Bird No. 271 | Bird No. 651 | Bird No. 833 | Bird No. 1028 | Bird No. 1043 |
| | Weeks of depletion → | 24 | 3 | 2 | 3 | 8 | 24 | 24 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 375 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 700 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 1400 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 2650 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 5475 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 8250 | trace | 0 | 0 | trace | 0 | trace | 0 |
| 8 | 12,500 | 16.5 | trace | trace | 10.9 | trace | 6.8 | 0 |
| 9 | 18,300 | 14.8 | 7.9 | 8.3 | 10.0 | 5.6 | 8.3 | trace |
| 10 | 24,800 | 15.6 | 6.9 | 5.4 | 9.3 | 4.7 | 4.9 | 2.9 |
| 11 | 38,500 | 17.5 | 6.9 | 6.1 | 25.6 | 4.4 | 7.8 | 3.3 |
| 12 | 46,200 | 12.1 | 6.0 | 6.0 | 19.3 | 3.9 | 8.7 | 4.8 |
| 13 | 57,000 | 12.2 | 9.1 | 7.8 | 12.0 | 4.4 | 5.3 | 3.8 |
| Average excretion % | | 14.8 | 7.4 | 6.7 | 14.5 | 4.6 | 7.0 | 3.7 |
| Average retention % | | 85.2 | 92.6 | 93.3 | 85.5 | 95.4 | 93.0 | 96.3 |

The percentage of vitamin A excreted appeared to be characteristic of each hen and remained of the same order for each throughout the experiment, although, of course, the actual quantity excreted increased with increasing dosage. Furthermore the length of the depletion period, whether 2 or 24 weeks, had no effect on either the percentage of vitamin A excreted or the first appearance of a detectable amount of the vitamin. It was first detected in the droppings of those birds which excreted the highest percentages. Thus, two of the first three birds to eliminate vitamin A had been on the depletion diet

for 24 weeks, and one, no. 1022, showed the highest percentage excretion of the group. These data indicate that any measurement of the efficiency of absorption of vitamin A would be of no value in determining the nutritional status of the hen. It is of interest to note that even after 24 weeks' depletion there were no gross symptoms of vitamin A deficiency and that the body weights of the birds did not change appreciably during the experiment.

Twenty-two hours after the close of the final period, vitamin A could not be detected in the excreta of either group and therefore it is concluded that the excretion for each period is essentially from the quantity of the vitamin fed during that period. The same observation was made 3 days later and therefore it is concluded that the vitamin is not eliminated from the body stores either through the kidneys or the intestine.

Two of the low fat birds showed a higher percentage, retention than did any of the four birds of the normal group and the average excretion of the low fat group was approximately half that of the normal birds. It is probable that this is not a significant difference because of the small number of individuals involved.

Each 200 units of vitamin A ester fed was contained in approximately 1 mg. of oil solvent. Later experiments with growing chicks (Polskin, '40), in which was studied the effect of fat on the absorption of crystalline carotene and of vitamin A alcohol, indicate that more of these factors reach the liver when fed with fat. In these preliminary studies 1 mg. of fat (corn oil) was administered with each 200 units of vitamin A (or carotene) as in the feeding of vitamin A ester in the present experiment, and the fat contents of the rations were essentially the same in both experiments. The total fat intake was raised slightly though probably not significantly by the supplements. In period 7, for example, in which traces of vitamin A first appeared in the droppings, the quantity of fat fed as the solvent raised the level of fat in the diet from 0.07 to 0.08%. Therefore, in the present experiment, the fatty

solvent of the vitamin A ester probably favored absorption of the factor, either by a protective role in the tract or by actually aiding in absorption, but the extent of its influence is not known.

The results on both the low fat and the normal rations show that the vitamin A ester is absorbed more completely than is carotene and suggest that vitamin A values calculated from the carotene content of the ration or based on rat assays may not be entirely reliable for the hen.

TABLE 4

*Vitamin A content of livers of hens on normal and low fat rations 1 month after receiving large doses of vitamin A.*

| GROUP | BIRD NO. | WT. OF LIVER | VITAMIN A PER GM. OF LIVER U.S.P. XI | TOTAL VITAMIN A IN LIVER U.S.P. XI | VITAMIN A RECOVERED IN LIVER |
|---|---|---|---|---|---|
| | | *gm.* | *units* | *units* | *%* |
| Normal | 1316 | 15.1 | 6875 | 94,380 | 43.7 |
| | 271 | 19.6 | 4650 | 91,140 | 42.4 |
| | 1022 | 19.0 | 3650 | 69,350 | 32.1 |
| | 651 | 53.7 | 1212 | 65,120 | 30.1 |
| Average | | | 4096 | 80,000 | 37.0 |
| Low fat | 1028 | 21.3 | 630 | 13,425 | 6.2 |
| | 833 | 20.0 | 490 | 9,850 | 4.5 |
| | 1043 | 20.3 | 420 | 8,525 | 3.9 |
| Average | | | 513 | 10,600 | 4.9 |

The hens were maintained on the respective rations for 1 month after the last dose of vitamin A was administered, after which they were killed and the quantity of the factor in the liver was determined. In table 4, it is noted that in the normal group the average vitamin A in the liver was 80,000 units, whereas in the low fat group, it was 10,000 units. The former value is 37% of the total number of units administered in the 13 periods and the latter only 4.9%. Since there was no difference between the normal and low fat group in their vitamin A excretions it was assumed that the liver storage of the factor was the same in both groups. This was not determined on the hens because of the small number available but in another experiment with growing chickens (Polskin, '40)

it was found that the liver content of vitamin A, following a feeding regime similar to that of the present experiment, was essentially the same on both normal and low fat rations. Therefore, assuming equal storage, it must be concluded that the presence of a normal amount of fat in the ration was accompanied by a much greater retention of vitamin A during the depletion period when no vitamin A was fed, than occurred in the case of the low fat ration.

### SUMMARY AND CONCLUSION

1. In hens the absorption of carotene in the crystalline form is improved by the presence of fat in the ration.

2. On the low fat ration, and presumably on the normal, there appears in the excreta a yellow pigment which has the solubility properties of carotene, but which, according to spectrophotometric determination, is not a member of the carotene group of pigments.

3. Neither carotene nor vitamin A is eliminated from the body stores by way of either the kidney or the intestine.

4. Absorption of vitamin A by hens was essentially the same on normal and low fat rations. The length of the depletion period, prior to feeding vitamin A, had no effect upon the percentage of the factor absorbed. As the feeding levels were increased, the percentage of the factor excreted remained essentially the same in both groups and was characteristic of the individual hen. The presence of a small quantity of fat as the vehicle for vitamin A ester (1 mg. per 200 units) may have favored absorption.

5. The retention of vitamin A in the liver was greater in the hens which received the ration with normal fat content.

### LITERATURE CITED

AHMAD, B.   1931   The fate of carotene after absorption in the animal organism. Biochem. J., vol. 25, p. 1195.

BASU, N. K.   1937   Vitamin A and fat metabolism. Zeitschr. fur Vitaminforsch., vol. 6, p. 106.

CARR, F. H., AND E. A. PRICE   1926   Color reaction attributed to vitamin A. Biochem. J., vol. 20, p. 497.

DE, N. K. 1937 The assimilation of vitamin A and carotene by rats from some common foods with a note on the conversion factor proposed by the International Vitamin Conference. Ind. J. Med. Res., vol. 24, p. 751.

FODOR, A., AND R. SCHOENFELD 1931 Darstellung und Eigenschaften wässeriger Carotinlösungen. Biochem. Z., vol. 233, p. 243.

GRAY, E. L., K. C. D. HICKMAN AND E. F. BROWN 1940 The state of vitamin A in the liver of the rat after feeding various forms of the vitamin. J. Nutrition, vol. 19, p. 39.

KEMMERER, A. R., AND G. S. FRAPS 1938 The digestibility of carotene by rats and chickens. J. Nutrition, vol. 16, p. 309.

PETERSON, W. J., J. S. HUGHES AND H. F. FREEMAN 1937 Determination of carotene in forage. Ind. Eng. Chem., Anal. Ed., vol. 9, p. 71.

PETERSON, W. J., J. S. HUGHES AND L. F. PAYNE 1939 The carotenoid pigments. Occurrence, properties, methods of determination, and metabolism by the hen. Kansas Agr. Exp. Sta., Tech. Bull. 46, 74 pp.

POLSKIN, L. J. 1940 Studies on the absorption and retention of vitamin A and carotene by the chicken on normal and low fat rations. Doctor's Thesis, Rutgers University, New Brunswick, New Jersey.

RECORD, P. R., R. M. BETHKE AND O. H. M. WILDER 1937 The vitamin A requirements of chicks with observations on the comparative efficiency of carotene and vitamin A. Poultry Sci., vol. 16, p. 25.

RUSSELL, W. C., M. W. TAYLOR AND L. J. POLSKIN 1940 Fat requirements of the growing chick. J. Nutrition, vol. 19, p. 555.

VAN EEKELEN, M., AND W. PANNEVIS 1938 Absorption of carotenoids from the human intestine. Nature, vol. 141, p. 203.

WILSON, H. E. C., S. M. DAS GUPTA AND B. AHMAD 1937 The absorption of carotene and vitamin A in the human subject. Ind. J. Med. Res., vol. 24, p. 807.

# THE EFFECTIVENESS OF LINOLEIC, ARACHIDONIC, AND LINOLENIC ACIDS IN REPRODUCTION AND LACTATION [1,2]

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In comparing individual fatty acids or their derivatives for biological activity, restoration of growth and prevention or cure of dermal lesions have served heretofore as the criteria (Burr and Burr, '30; Burr, Burr and Miller, '31; Turpeinen, '38; Hume et al., '40). Although reproduction is seriously impaired in fat deficiency, little is known concerning the unsaturated acid requirements for this important biological function. Normal litters of rats have been produced with dietary supplements of natural fats such as lard and butterfat (Burr and Burr, '30; Evans et al., '34; Maeder, '37), and inferior litters with linoleic acid either as a concentrate (Evans et al., '34), or as the pure methyl ester (Mackenzie et al., '39). Data concerning other acids or esters are lacking.

In experiments with low-fat diets most workers have used ether-extracted yeast as the source of the vitamin B complex (Burr and Burr, '30; Evans et al., '34). The use of this material is open to criticism since it has been shown that yeast contains from 2.5 to 4% of lipids which are firmly bound and non-extractable by ether (Smedley-Maclean, '22; Newman and Anderson, '33). Mackenzie, Mackenzie and McCollum

('39) have recently used a water-soluble extract from yeast, but unfortunately, they fed a relatively low level of methyl linolate.

Recent work by Schneider and Steenbock ('39) had shown that a commercially available water-soluble rice bran concentrate very low in lipids, constituted a good source of the vitamin B complex when fed in a diet which contained egg white as the source of protein. In the present work this product was fortified with riboflavin to make a satisfactory diet in which purified casein served as the protein. In parallel experiments using this diet of extremely low fat content and a diet containing ether extracted yeast, unsaturated acids of high purity were compared for their nutritive value.

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EXPERIMENTAL

*Diets*

</div>

The first diet, designated the "Rice-Extract Diet," contained glucose [3] and a rice bran concentrate [4] with added riboflavin; the second, which was a duplication of the Burr and Burr diet, designated the "Yeast Diet," contained sucrose and ether-extracted yeast (table 1).

Both diets were supplemented with 100 µg. carotene, 35 µg. calciferol, 350 µg. tocopherol [5] and 100 µg. vitamin $K_1$ [5] per rat per week. These supplements were dissolved in a liquid fraction of hydrogenated coconut oil (I. V. = 0.1), and 1 drop of the solution was administered twice weekly. The flavin was dissolved in N/50 acetic acid, mixed with the rice bran concentrate and incorporated in the ration. The yeast was fed in small dishes.

Although the yeast had been extracted with ethyl ether in a continuous extractor for 3 days, the daily intake of unsaturated lipid from yeast was many times as high as that from the rice bran concentrate (table 2). This was revealed

[3] Cerelose.

[4] Vitab Type II, National Oil Products Company.

[5] We are indebted to Merck and Company, Inc. for the α-tocopherol and vitamin $K_1$.

by treatment of both desiccated and moist samples of the two
materials with acidified alcohol and subsequent extraction of
the products. In the first case 100 gm. of yeast or rice bran
concentrate were dried over $P_2O_5$ and refluxed for 2 hours

TABLE 1

*Composition of diets.*

|  | RICE-EXTRACT DIET | YEAST DIET |
|---|---|---|
| Casein (alcohol-extracted)[1] | 18 | 18 |
| Wesson salts | 4 | 4 |
| Cerelose | 75 | .. |
| Sucrose | .. | 72 |
| Ether-extracted yeast [2] | .. | 0.65 gm./day |
| Rice bran concentrate [3] + 2 mg. riboflavin/kg. ration | 3 | .. |

[1] Labor for the purification of the casein was furnished by the Works Progress Administration.
[2] Brewers yeast, Anheuser-Busch Company.
[3] Vitab type II, National Oil Products Company.

TABLE 2

*Lipid content of dietary constituents by cleavage with HCl.*

| CONSTITUENT | TOTAL LIPID (P. E. EXTRACT) | IODINE VALUE | APPPROXIMATE DAILY LIPID INTAKE [1] | |
|---|---|---|---|---|
|  |  |  | Total | Unsaturated (calcd. as linoleic) |
|  | % |  | mg. | mg. |
| Yeast (desiccated) | 2.43 | 85.7 | 16.0 | 7.6 |
| Rice bran concentrate (desiccated) | 0.36 | 64.0 | 1.1 | 0.4 |
| Yeast (moist) | 4.21 | 84.7 | 27.0 | 12.6 |
| Rice bran concentrate (moist) | 0.44 | 41.6 | 1.3 | 0.3 |
| Casein | 0.11 | 40.4 | 2.0 | 0.4 |

[1] Based on an assumed consumption of 10 gm. of diet daily.

with 300 cc. of a solution of 3% dry HCl in absolute alcohol.
The extract was diluted with an equal volume of water and
shaken with three 100 cc. portions of chloroform. The alcohol-
insoluble residue was extracted with chloroform for 2 days

in a Soxhlet. The chloroform extracts were combined, washed thoroughly with water, dried over sodium sulphate and freed from chloroform under reduced pressure. The residue was taken up in 5 to 10 cc. of chloroform and freed from non-lipid substances by precipitation with petroleum ether. The petroleum ether-soluble material was washed with water, dried and freed from solvent.

Hydrolysis with 3% HCl in 80% alcohol produced even a larger yield of lipid from the yeast. The procedure was identical with that detailed above except that before refluxing, 20% of water was added to the yeast to make it equivalent in water content to the rice bran concentrate.

The casein was purified by extraction with warm alcohol (50°C.). Nevertheless, it yielded a small amount of lipid on acid hydrolysis (table 2). For the hydrolysis 100 gm. were heated with 300 cc. of dilute HCl (1:3) for 20 hours on a steam bath. The solution was cooled, treated with an additional 25 cc. of HCl and extracted with three 100 cc. portions of redistilled petroleum ether. The combined extracts were washed with three 100 cc. portions of water and dried over sodium sulphate. The solvent was removed under reduced pressure and the residue weighed, giving a yield of 90 mg. When the aqueous phase was refluxed vigorously for 6 hours more, an additional 20 mg. of lipid were obtained giving a total of 110 mg.

Based on these analyses, the yeast furnished 27 mg. of lipid per rat daily. Assuming an average food intake of 10 gm. daily the rice bran concentrate furnished 1.3 mg. of lipid. In terms of unsaturated lipid these differences were even greater. Expressing the unsaturated compounds in terms of linoleic acid, a maximum of 12.6 mg. of linoleic acid was furnished by the yeast and 0.4 mg. by the rice bran concentrate. Adding the lipid which was supplied by the casein these values become 13.0 and 0.8 mg. for the yeast and rice-extract diets, respectively.

The fatty acids were fed as the ethyl esters. The ethyl linolate and linolenate were prepared from corn oil and

linseed oil by a modification of the Rollett method ('09). The ethyl arachidonate was prepared from beef suprarenal phosphatide [6] by low temperature crystallization and fractional distillation (Shinowara and Brown, '40). The distilled ethyl esters had the following iodine values (Wijs, 4 hr.): ethyl linolate 166 (theor. 164.8); ethyl linolenate 245 (theor. 248.9); ethyl arachidonate 299 (theor. 305.5).

## Preparation of animals

To minimize the pre-experimental storage of essential unsaturated fatty acids, litters at 12 days of age were transferred with their mothers to a diet consisting largely of potatoes (Quackenbush et al., '39). The young were weaned when they weighed 40 gm. and placed in individual metal cages where they consumed the experimental diets ad libitum. Thirty female rats were given the rice-extract diet, and twelve females and fourteen males the yeast diet. After 12 weeks the females on each diet were divided into five groups. The heaviest animals, weighing about 178 gm., received only the basal diet; the lightest, which averaged 131 gm., received in addition supplements of cottonseed oil. The remainder were divided equally among the other groups. Three weeks later all were mated with normal males. After the finding of sperm in the vaginal smear the allowances of fat-soluble vitamins and vitamin B complex were doubled. The litters were reduced to six young each, 24 hours after parturition. During lactation the dose of the vitamin B complex was tripled.

### RESULTS

## Growth and general appearance

Animals fed the low-fat diets grew to maturity, but at the twelfth week their body weights were significantly less than those of comparable animals fed our stock diet (Steenbock, '23). Those getting the rice-extract diet weighed 151 gm. and

[6] We are indebted to Dr. O. Kamm of Parke-Davis and Company for this preparation.

those receiving the yeast diet 154 gm., while females in the stock colony weighed 195 gm. at the same age.

The addition of various lipid supplements after 12 weeks on either of the two basal diets resulted in growth, the magnitude of which depended upon the nature of the supplement as well as on the basal ration. In 3 weeks cottonseed oil produced a gain of 50 gm. in the animals ingesting the rice-extract diet and 30 gm. in those fed the yeast diet. Ethyl linolate produced a gain of 26 gm. on either diet, while ethyl arachidonate produced a gain of 34 gm. on the rice-extract diet and 19 gm. on the yeast diet. Ethyl linolenate produced much smaller gains, viz., 14 gm. on the rice-extract diet and only 8 gm. on the yeast diet. Without supplements the animals on the former diet gained slightly, viz., 7 gm.; those on the latter diet did not gain. A further demonstration of the necessity of fatty acids for growth was obtained from two groups of animals which had received ethyl linolate or cottonseed oil beginning with the second instead of the twelfth week on the rice-extract diet. Those receiving ethyl linolate weighed 188 gm. and those getting cottonseed oil weighed 195 gm. at the end of the twelfth week.

The animals on either of the low-fat diets developed a scaliness of the hind paws and tail after they had been on the experimental diet from 9 to 12 weeks. No scaliness or loss of hair around the eyes, nose or mouth was noted. Within 3 weeks after supplements of cottonseed oil, ethyl linolate or ethyl arachidonate were given, the scaliness of the hind paws and tail was cured completely. The cured animals were sleek and in every way appeared like stock rats. However, with ethyl linolenate the scaly condition of the hind paws and tail persisted even after 7 weeks.

## Reproduction and lactation

A failure of normal parturition always ensued on the basal diets (table 3). Labor began at term, but parturition was accompanied by excessive hemorrhage and was not completed for 2 to 3 days. The animals lost weight and became extremely

weak and anemic. Two of the females died before giving birth to their young. All of the young died within 48 hours after birth.

When supplements of cottonseed oil, ethyl linolate, or ethyl arachidonate were fed, the needs of both parturition and lactation were met. The rice-extract diet gave approximately the same results as the yeast diet with the same fat supple-

TABLE 3

*The effects of fatty acid esters on reproduction.*

| GROUP | SUPPLEMENT | AMOUNT FED DAILY | RICE-EXTRACT DIET | | | | | YEAST DIET | |
|---|---|---|---|---|---|---|---|---|---|
| | | | No. of rats | Gestation period | Mean no. young per litter | Born alive | Weaned | Born alive | Weaned |
| | *12th to 19th week:* | *drops* | | *days* | | % | % | % | % |
| 1 | None | .. | 6 | 25 | 4 | 8 | 0 | 73 | 0 |
| 2 | Ethyl linolate | 5 | 5 | 22 | 6 | 100 | 34 | 100 | 83 |
| 3 | Ethyl linolenate | 5 | 5 | 24 | 3 | 56 | 0 | 83 | 0 |
| 4 | Ethyl arachidonate | 5 | 5 | 22 | 9 | 100 | 67 | 100 | 55 |
| 5 | Cottonseed oil | 10 | 2 | 22 | 5 | 100 | 80 | 100 | 92 |
| | *2nd to 19th week:* | | | | | | | | |
| 6 | Ethyl linolate | 5 | 5 | 22 | 6 | 100 | 79 | .. | .. |
| 7 | Cottonseed oil | 5% in diet | 2 | 22 | 6 | 100 | 89 | .. | .. |

ments. When ethyl linolate was given after the second week of the experimental period, the results compared favorably with those obtained on our stock diet.

By way of contrast ethyl linolenate produced results only slightly better than those obtained on the basal diet. While the gestation period was prolonged and vaginal bleeding was the same as on the basal diets, three of the fourteen young born alive in group 3, survived the first week. One of these reached a weight of 40 gm. at 5 weeks and the other two a weight of 31 gm. at 4 weeks of age, but all died shortly thereafter. All had scaly feet and tails, in contrast with the young receiving the other lipid supplements, which were entirely normal.

## Other fat deficiency symptoms

Failure of oestrus as reported by Burr and Burr ('30) and Evans et al. ('34) was not observed in our animals during 20 weeks on low-fat diets. Pseudo pregnancies appeared in two of our deficient animals. This defect was not corrected by ethyl linolate. Resorptions as reported by Maeder ('37) were not observed in any of our animals.

A comparison of the amount of water consumed did not reveal striking differences. During the twentieth week females weighing 190 to 200 gm. which received the rice-extract diet with and without fat consumed 27 and 28 cc. of water daily; those fed the yeast diet consumed 26 and 30 cc., respectively; and stock rats weighing 221 gm. consumed 30 cc. However, 28 weeks later males on the yeast diet consumed 47 cc. of water instead of 36 in spite of a drop in weight from 204 to 176 gm.

The scaliness of the hind paws and tail increased with the continued feeding of the yeast diet. At 48 weeks the hind paws were extremely scaly and somewhat erythematous; the tail was very scaly and ringed; the ears were thickened, and the testes had atrophied. Supplements of pyridoxine, pantothenic acid, or rice bran concentrate did not alleviate the symptoms. The animals became scrawny and listless and most of them died. At autopsy, kidney stones were found in 20% of them. Yet at 62 weeks four of the original fourteen male rats were still alive. Two of these had hematuria. Rice bran concentrate did not correct this but the hematuria did disappear after the administration of cottonseed oil.

## Fat analyses

Fat analyses were made on the females and on all young which had reached a weight of 40 gm. The animals were killed with ether and analyzed according to the procedure described by Quackenbush and Steenbock (in press). The liver and carcass were analyzed separately (table 4).

The animals on the yeast diet contained less fat than those on the rice-extract diet, but the iodine number was higher.

However, the trend of results after feeding individual fatty acids was the same as on the rice-extract diet. The group receiving ethyl linolenate did not differ in fat content nor in iodine value from those which had received the essential fatty acids. Although unsaturated fat was evidently synthesized by the animals which did not receive dietary fat, and also by those which received ethyl linolenate, it was apparently not of an essential character.

TABLE 4

*Analyses of body fats of adult female rats.*

| GROUP | SUPPLEMENT | RICE-EXTRACT DIET | | | YEAST DIET | | |
|---|---|---|---|---|---|---|---|
| | | Mean body weight | Total fat | Iodine number | Mean body weight | Total fat | Iodine number |
| | | *gm.* | % | | *gm.* | % | |
| 1 | No fat | 202 | 11.0 | 67 | 186 | ... | .. |
| 2 | Cottonseed oil | 213 | 10.8 | 69 | 169 | 8.9 | 74 |
| 3 | Ethyl linolate | 196 | 10.7 | 66 | 201 | 7.6 | 75 |
| 4 | Ethyl linolenate | 192 | 10.3 | 70 | 196 | 8.3 | 72 |
| 5 | Ethyl arachidonate | 204 | 11.4 | 65 | 185 | 8.1 | 70 |
| 7 | Ethyl linolate from 2nd week | 208 | 10 4 | 68 | ... | ... | .. |

The analyses of the body fats and liver fats of the young produced on the two diets likewise revealed no significant differences. The body fats ranged from 4.8 to 7.1% with iodine numbers from 60 to 78, and liver fats from 1.3 to 3.9% with iodine numbers from 77 to 112. The higher iodine numbers were found in the groups of the lower fat content.

### DISCUSSION

The rice-extract diet apparently was superior to the yeast diet because it gave the better growth after the addition of essential fatty acids. This is not inconsistent with the observation of Schneider et al. ('40). However, in view of the results obtained in our long-term experiments, the interpretation of Schneider et al. ('40), that rice bran concentrate can replace the essential fatty acids in all their functions, is not sub-

stantiated. Rice bran concentrate can prevent acrodynia but it cannot prevent the scaliness of the feet and tail.

On the basal diets, failure of reproduction always ensued. After a prolonged gestation period, parturition was accompanied by excessive vaginal bleeding and loss in weight. These results are in agreement with the observations of Maeder ('37) that hemorrhages in the placenta and uterine wall are the cause of fetal death in fat deficiency.

The low percentage and the poor nutritive state of the young weaned in the experiments of others with linoleic supplements were probably due to the low levels fed. Evans et al. ('34) supplemented a yeast diet with 40 mg. of linoleic acid in the form of a concentrate from corn oil. Although this dose was increased to 120 mg. after parturition, only half of the young survived, and those weighed only 28 gm. at weaning. When 25% of lard was included in the diet over 90% survived, and the weight at weaning was 39 gm. Mackenzie, Mackenzie and McCollum ('39) fed 25 mg. of methyl linolate with a diet containing a water-soluble extract of yeast and carried their female rats through three gestation periods. No young were weaned until the second and third gestations and the young of the heaviest litter averaged 31.0 gm. at 21 days. It is possible that the level of methyl linolate was inadequate for the first gestation, but due to a gradual and tenacious storage (Sinclair, '35) sufficient amounts of the essential fatty acids accumulated for the later gestations. In view of these results it is recommended that in reproduction and lactation studies rats should receive daily at least 100 mg. of the essential fatty acids or their equivalent in the form of a natural oil. Turpeinen ('38) has already recommended the use of 100 mg. for maximal growth.

Maeder ('37) has stated that "failure of reproduction in humans due to lack of adequate fat in the diet is considered possible." It is not excluded that reported beneficial effects of wheat germ oil in human pregnancy may have been due in part to meeting the essential fatty acid requirements.

The results of the reproduction studies parallel those obtained when dermal lesions were used as criteria. When assayed with acrodynic rats as described by Quackenbush et al. ('39), 10 mg. daily of either linoleic or arachidonic acid cured the dermal symptoms. As these animals weighed 50 gm. and our females weighed 200 gm., the requirement per kilogram of body weight is about twice as high for reproduction as for the cure of dermal lesions. Linolenic acid was ineffective in both instances. Burr, Brown, Kass and Lundberg ('40) have reported that linolenic acid gave good growth responses but had little effect on the skin. Arachidonic and linoleic acids were similar to each other in their effects. Our data on reproduction as well as on acrodynia have revealed no significant difference of physiological activity between linoleic and arachidonic acids; however, they corroborate abundantly the practical inability of linolenic acid to correct the symptoms of fat deficiency (Hume et al., '38).

## CONCLUSIONS

1. On a low-fat diet furnishing only 3.0 mg. of unsaturated lipid or a calculated maximum of 0.8 mg. of linoleic acid per rat per day, rats were raised to maturity and bred. After a prolonged gestation period and severe hemorrhage in parturition, the young were born dead or died soon after birth. A scaly condition of the hind paws and tail was observed after about 10 weeks on the diet.

2. Ethyl linolate and ethyl arachidonate prevented or cured the dermal symptoms completely and produced normal young which were weaned at the age of 3 weeks with an average body weight of 40 gm. The requirement for these acids appears to be higher than previously estimated.

3. Ethyl linolenate did not make possible the production of normal young; neither did it cure the dermal symptoms.

4. Fat analyses revealed a remarkable constancy in both the percentage of total fat and the iodine values of the fat, irrespective of the dietary supplements.

## LITERATURE CITED

BURR, G. O., AND M. M. BURR 1930 On the nature and role of the fatty acids essential in nutrition. J. Biol. Chem., vol. 86, p. 587.

BURR, G. O., M. M. BURR AND E. S. MILLER 1931 On the fatty acids essential in nutrition. J. Biol. Chem., vol. 97, p. 1.

BURR, G. O., J. B. BROWN, J. P. KASS AND W. O. LUNDBERG 1940 Comparative curative values of unsaturated fatty acids in fat deficiency. Proc. Soc. Exp. Biol. Med., vol. 44, p. 242.

EVANS, H. M., S. LEPKOVSKY AND E. A. MURPHY 1934 Vital need of the body for certain unsaturated fatty acids. J. Biol. Chem., vol. 106, p. 431.

HUME, E. M., L. C. A. NUNN, I. SMEDLEY-MACLEAN AND H. H. SMITH 1940 Fat-deficiency disease of rats. Biochem. J., vol. 34, p. 879.

——————— 1938 Studies of the essential unsaturated fatty acids in their relation to the fat-deficiency disease of rats. Biochem. J., vol. 32, p. 2162.

MACKENZIE, C. G., J. B. MACKENZIE AND E. V. MCCOLLUM 1939 Growth and reproduction on a low-fat diet. Biochem. J., vol. 33, p. 935.

MAEDER, E. C. 1937 The effect of fat in simplified diets on the reproductive organs of the female albino rat during gestation. Anatomical Record, vol. 70, p. 73.

NEWMAN, M. S., AND R. J. ANDERSON 1933 The chemistry of the lipids of yeast. 1. The composition of the acetone-soluble fat. J. Biol. Chem., vol. 102, p. 219.

QUACKENBUSH, F. W., B. R. PLATZ, AND H. STEENBOCK 1939 Rat acrodynia and the essential fatty acids. J. Nutrition, vol. 17, p. 115.

QUACKENBUSH, F. W., AND H. STEENBOCK Body fats in rat acrodynia. (J. Nutrition, in press.)

ROLLETT, A. 1909 Zur Kenntnis der Linolsaure. Zeitschrift f. Physiol. Chemie., vol. 62, p. 411.

SCHNEIDER, H., AND H. STEENBOCK 1939 A low phosphorus diet and the response of rats to vitamin D$_2$. J. Biol. Chem., vol. 128, p. 159.

SCHNEIDER, H., H. STEENBOCK AND B. R. PLATZ 1940 Essential fatty acids, vitamin B$_6$, and other factors in the cure of rat acrodynia. J. Biol. Chem., vol. 132, p. 539.

SHINOWARA, G. Y., AND J. B. BROWN 1940 Study on the chemistry of fatty acids. VI. The application of crystallization methods to the isolation of arachidonic acid, with a comparison of the properties of this acid prepared by crystallization and by debromination. Observations on the structure of arachidonic acid. J. Biol. Chem., vol. 134, p. 331.

SINCLAIR, R. G. 1935 The metabolism of the phospholipids. VII. Further evidence of the selection and retention of unsaturated fatty acids by phospholipids of animal tissues. J. Biol. Chem., vol. 111, p. 275.

SMEDLEY-MACLEAN, I. 1922 The conditions influencing the formation of fat by the yeast cell. Biochem. J., vol. 16, p. 370.

STEENBOCK, H. 1923 A satisfactory ration for stock rats. Science, vol. 58, p. 449.

TURPEINEN, O. 1938 Further studies on the unsaturated fatty acids essential in nutrition. J. Nutrition, vol. 15, p. 351.

# LINOLEIC ACID, PYRIDOXINE AND PANTOTHENIC ACID IN RAT DERMATITIS [1, 2]

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Studies in numerous laboratories have shown that rat dermatitis in its various forms is etiologically related to a number of deficiencies. The work of Goldberger and Lillie ('26) emphasized the importance of a water-soluble substance in preventing or curing dermal lesions. The findings of Burr and Burr ('29) focused attention on fats and constituent fatty acids. Richardson and Hogan ('36), Birch and György ('36) and Salmon ('38) demonstrated that water-soluble and lipid factors were both involved in the prevention and cure of the severe form of dermatitis now frequently termed "rat acrodynia." Tests with isolated compounds have shown that linoleic acid (Quackenbush et al. '39), pyridoxine (György, '38; Lepkovsky, '38), and pantothenic acid (György et al., '39) are factors involved in the production and cure of the disease. These findings have been substantiated by other investigators. However, the effects of the individual factors have not been clearly demonstrated since the basal diets usually contained considerable quantities of either unsaturated fat or crude concentrates of the water-soluble factors. The present report deals with the interrelationships observed when the afore-

mentioned supplements were given as pure compounds super-imposed on a basal diet lacking all of them.

## EXPERIMENTAL

All the experiments were performed with rats. In preparation for experiment, litters were raised to weaning age on a diet consisting chiefly of potato meal (Quackenbush et al., '39). They were weaned at a weight of 38 to 42 gm. and placed on the basal diet (diet 5) or, in some of the prophylactic studies, on a modification of this diet. Diet 5 had a percentage composition of glucose[3] 78, casein[4] (alcohol-extracted) 18, and salts[5] 4. In addition each rat was given a daily dose of 10 µg. of thiamine and 20 µg. of riboflavin[6] dissolved in 1 drop of 0.02 N acetic acid, and 10 µg. of carotene and 5 µg. of calciferol dissolved in 1 drop of liquid hydrogenated coconut oil (I. V. = 0.1). The casein carried a small amount of unsaturated lipid (0.11%, I. V. = 40.0) extractable after acid hydrolysis. In all, the basal diet contained no more than 0.003% of unsaturated lipid calculated as linoleic acid.

The test supplements were administered by dropper. Pyridoxine hydrochloride[6] and calcium d-pantothenate[6] were fed as synthetic compounds in aqueous solution. The ethyl linolate was prepared from corn oil via the tetrabromide (m. p. 115.0°). The freshly distilled ester (I. V., Wijs, 164.0) was sealed in glass ampules under high vacuum. After an ampule was opened for use its contents were kept under nitrogen in a refrigerator between feedings. A fresh supply was provided from a new ampule every 3 or 4 days.

In evaluating the effects of the various supplements, attention was given not only to the acrodynia in its various stages but to all phases of erythematous · dermatitis as well as

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[3] Cerelose.

[4] Labor for the purification of the casein was furnished by the Works Progress Administration.

[5] Wesson.

[6] The pyridoxine, pantothenic acid and riboflavin were generously supplied by Merck and Company.

to the dry scaliness which often persisted after all evidence of inflammation had disappeared. The term "acrodynia" was applied to both the acute form (Quackenbush et al., '39) characteristic of animals on our basal diet, and the chronic form of "rat acrodynia" (Birch et al., '35) which is produced on diets partially deficient in the essential fatty acids. The chronic form developed less rapidly, involved larger areas of the peripherae, and lacked the topographical uniformity of distribution as well as the severity of the acute form. The term "dry scaliness' was applied to cases of exfoliation in which erythema and exudation were not apparent. This condition resembled that which develops on a low-fat diet containing yeast or rice bran concentrate (Burr and Burr, '29; Quackenbush et al., '42), and which is commonly attributed to a fat deficiency.

To facilitate comparison of the degree of dermal involvement, initially and during the progress of the tests, the extent and severity of the lesions were expressed as a dermal index. This index was a numerical expression of the stage of development of the lesions of the lips, eyelids, fore-paws, hind-paws, ears and tail. It was obtained by recording the stage of development at each of these foci in accordance with our previous method (Quackenbush et al., '39) and adding the recorded numbers. The sum, or dermal index, had a theoretical maximum of 23. In practice the highest observed before death was 16.

### Therapeutic relations

The weanling rats placed on the basal diet developed a severe acrodynia in 4 to 5 weeks. The test supplements were administered when the animals had a dermal index of 5 to 6, at which time the body weight usually ranged from 50 to 60 gm. Daily supplementation was continued for a period of 3 or more weeks. A cure of acrodynia was assumed to have been effected when the lips, eyes and ears were normal in appearance and the paws and tail free from all symptoms except for a dry scaliness, i.e., a dermal index of 1 or 2.

The therapeutic results obtained with both single and multiple supplements are summarized in table 1.

When fed alone, pantothenic acid produced no improvement. Pyridoxine tended to arrest the development of acute symptoms; it extended the period of survival and thereby permitted the chronic form to develop. An early improvement in the condition of the lips and fore-paws was followed by an intensification of dermal lesions of both the hind- and fore-paws. Lesions also appeared on the ears and tail.

TABLE 1

*Curative effects of single and multiple supplements.*

| | DAILY SUPPLEMENT | | | NO. OF RATS | MEAN 3-WEEK GAIN [1] | MEAN DERMAL INDEX AT | | |
|---|---|---|---|---|---|---|---|---|
| Group | Ethyl linolate | Pyri-doxine | Panto-thenic acid | | | Start | 3-weeks [1] | 6 weeks [1] |
| | *mg.* | *μg.* | *μg.* | | *gm.* | | | |
| 1 | .. | .. | .. | 10 | —7 | 5 | 11 | .... |
| 2 | .. | 2 | .. | 4 | 4 | 5 | 6 | 8 (4) |
| 3 | .. | 4 | .. | 4 | 5 | 5 | 4 | 5 (3) |
| 4 | .. | 6 | .. | 4 | 13 | 6 | 7 | 5 (3) |
| 5 | .. | 10 | .. | 4 | 3 | 5 | 5 | 6 (4) |
| 6 | .. | 20 | .. | 68 | 6 | 6 | 7 | 6 (9) |
| 7 | .. | .. | 50 | 11 | —4 | 5 | 13 | .... |
| 8 | 5 | .. | .. | 11 | —2 | 6 | 6 | .... |
| 9 | 10 | .. | .. | 19 | 0 | 6 | 1 | 1 |
| 10 | 160 | .. | .. | 5 | 2 | 5 | 2 | .... |
| 11 | .. | 20 | 50 | 16 | 26 | 5 | 4 | 5 (4) |
| 12 | 10 | 20 | .. | 6 | 10 | 6 | 2 | .... |
| 13 | 10 | .. | 50 | 3 | 11 | 6 | 3 | 1 (3) |
| 14 | 10 | 10 | 50 | 3 | 59 | 7 | 2 | 2 (3) |
| 15 | 6 | 6 | 50 | 3 | 66 | 5 | 2 | 2 (3) |
| 16 | 5 | 5 | 50 | 6 | 29 | 5 | 2 | .... |
| 17 | 5 | 5 | .. | 5 | 5 | 5 | 2 | .... |
| 18 | 4 | 4 | 50 | 4 | 51 | 6 | 2 | 2 (4) |
| 19 | 2 | 2 | 50 | 4 | 30 | 5 | 3 | 3 (4) |
| Rice bran concentrate [2] | | | | | | | | |
| 20 | 200 | .. | .. | 13 | 33 | 5 | 2 | .... |

[1] The figures include only those animals which survived the full period. After the third week some animals were taken for other experiments, especially from the larger groups; hence in the data for 6 weeks effective totals are given in parentheses.

[2] Vitab.

Linoleic acid when fed as the ethyl ester at a subcurative level, viz., 5 mg. daily, produced an effect similar to that obtained with pyridoxine. When fed at higher levels, viz., 10 mg. or more, the acrodynia was cured completely. The final dermal index of 1 or 2 was due to a dry scaliness of the tail and, in some cases, of the hind-paws.

Pyridoxine together with pantothenic acid produced a greater improvement than was obtained with pyridoxine alone, but less than linoleic ester or a commercially available rice bran concentrate [7]. The dermal symptoms commonly persistent after this supplement were a narrow, denuded, slightly swollen erythematous margin surrounding the lips; a similar denuded, slightly scaly margin about the eyelids, thus sometimes giving a "spectacled" appearance; a dry scaliness on the dorsal side of the paws, with a slight erythema between digits; scaliness of the ears which were shriveled in appearance and shiny in exfoliated areas; and a scaliness of the tail, with ringlets, especially near the tip. Five drops of cottonseed oil daily cured these dermal symptoms except for a persistent dry scaliness of the hind paws and tail.

Ethyl linolate together with pyridoxine and pantothenic acid (group 14) cured the acrodynia completely, but a dry scaliness persisted just as it did when cottonseed oil was given with these water-soluble supplements.

When linoleic ester was fed in amounts insufficient to reduce the dermal index, the addition of pyridoxine brought about a definite improvement (groups 15 to 19). This result was not enhanced by the addition of pantothenic acid. These data leave no doubt concerning the existence of a supplementary effect between linoleic acid and pyridoxine.

From the above findings emerges the outstanding fact that for the cure of acrodynia under our experimental conditions linoleic acid was the primary indispensable curative factor. When present in sufficient amounts it cured the acrodynia without the addition of pyridoxine; when present in subcurative amounts its effectiveness was increased by the addition of pyridoxine.

[7] Vitab.

## Prophylactic relations

To determine the effect of various factors on the prevention of dermal lesions, five groups of rats were given supplements to the basal diet prior to the development of dermal abnormalities (table 2). The supplements were given from the time of weaning to all animals except those in group 23 which received the supplements after the third week.

Twenty or 40 μg. of pyridoxine daily produced a marked retardation in the development of lesions. None of the animals developed a severe acrodynia within 5 weeks, and many had

TABLE 2

*Effectiveness of dietary factors in preventing dermal lesions.*

| GROUP | DIET V ALTERATIONS | | | | NO OF RATS | MEAN DERMAL INDEX AT | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | pyri-doxine | panto-thenic acid | corn oil | casein | | 5th week | 12th week | 16th week |
| | μg. | μg. | % | % | | | | |
| 21 | 20 | .. | .. | .. | 27 | 2 | .. | .. |
| 22 | .. | 50 | .. | .. | 10 | 5 | .. | .. |
| 23 | 20 | 50 | .. | .. | 4 | 1 | 5 | .. |
| 24 | .. | 50 | 3 | .. | 10 | 0 | 1 | 2 |
| 25 | .. | 50 | 3 | 9 | 10 | 0 | 1 | 1 |

not attained a dermal index of 6 by the ninth week. During this period the mortality rate was high and a considerable number of rats succumbed without developing acrodynia. In general, the time required to produce severe lesions was longer than that required to obtain comparable lesions in relapsed animals in the curative series. Growth during the first 5 weeks was only slightly better than that of animals on the basal diet.

In marked contrast to pyridoxine, pantothenic acid did not retard the development of acrodynia. However, the two together retarded the development of symptoms more effectively than pyridoxine alone. The dermal symptoms were indistinguishable from those described for animals which received therapeutically pyridoxine together with pantothenic acid.

On diets lacking pyridoxine but supplying linoleic acid (corn oil) and pantothenic acid, animals did not develop acrodynia but grew poorly. At the twelfth week the mean body weights of rats in groups 24 and 25 were 88 and 72 gm., respectively; thereafter no appreciable growth resulted in either group. At the sixteenth week three animals in group 24 and one in group 25 showed a dry scaliness of the ears, hind-paws and tail. Thereafter, the mortality rate was high.

<div align="center">DISCUSSION</div>

Essential fatty acids hold a dominant position in acrodynia since when fed alone they can prevent or cure it. However, in the maintenance of a normal dermal condition other factors including pyridoxine, pantothenic acid, and at least one as yet unidentified factor are operative. This is evidenced in a number of ways: Pyridoxine can delay the appearance of symptoms and in some cases produce temporary healing. It can increase the efficiency of linolate to diminish the amount required to cure acrodynia. Pyridoxine together with pantothenic acid can alleviate or prevent the development of severe dermal lesions on a diet virtually free from essential fatty acids. Rice bran concentrate, supplying still other substances, can limit the dermal lesions to a mild dry scaliness.

Our preliminary report (Quackenbush et al., '41) concerning these experiments appeared simultaneously with reports from other laboratories (Salmon, '41; Richardson and Hogan, '41). Their conclusions, while for the most part in accord with ours, differ with respect to the curative effect of essential fatty acids when both pyridoxine and pantothenic acid are lacking. Richardson, Hogan and Itschner ('41) recently published their results in detail. They found that the dermatitis produced in rats fed diets virtually free from fat was not cured by 5 mg. of either linoleic acid or methyl arachidonate daily, and that variable results were obtained with higher levels of these supplements. The ineffectiveness of 5 mg. of either compound is in agreement with our results. Likewise the unreliability of free linoleic acid as a curative agent, even

when given in larger doses, is in agreement with our earlier observations (Quackenbush et al., '39). Their results with methyl arachidonate are of questionable significance since only two rats were given this supplement at levels higher than 5 mg. One of these was fed 10 mg. together with pyridoxine; it gave no response to either supplement. The other, which was fed 50 mg., was stated to have healed temporarily.

The results obtained by Richardson et al. ('41) with pyridoxine together with pantothenic acid also agree essentially with our data in respect to the persistence of the dermal symptoms. They reported that rats which were cured when given either linoleic acid or methyl arachidonate later developed dermatitis. We also observed mild erythematous lesions in one rat (group 25) and have previously observed relapses in four out of thirty-six rats from 12 to 15 weeks after they had been cured with oil supplements. However, these four rats failed to respond when given pyridoxine together with pantothenic acid, and the possibility is not excluded that after a multiple deficiency of such long standing, the animals were in a physiological state resistant to therapeutic measures.

The curative action of certain natural fats and purified esters of linoleic and arachidonic acids has been observed in a large number of animals. Our experience has shown that greater precautions must be taken to obtain consistent results with purified esters than with natural oils. The simple esters, unlike glycerides, have a tendency to cause loss of hair when spilled on the cheeks and chin; hence, care must be taken to place the supplement directly into the mouth if this is to be avoided. The purified esters contain no antioxidant and consequently oxidize rapidly. Esters having an appreciable peroxide number are not well received by the rat and are not as effective as the fresh material. Free fatty acids are exceedingly irritating.

Other important factors in determining the response of the rat to supplements are the stage of acrodynia and the weight of the animals at the time therapy is begun. Rats with a dermal index of 5 or 6 usually respond to potent supplements,

but if the index is allowed to advance beyond 6 the probability of response diminishes. Similarly, rats weighing 50 gm. or more show a higher percentage of response than rats of similar age weighing 35 or 40 gm. In therapeutic studies we have seldom used acrodynic animals with body weights of less than 50 gm.

The relationships of the different factors to growth have received little consideration in the present discussion. While each has been shown to have demonstrable effects, by far the greatest growth effects of single supplements were produced by the water-soluble factors.

All the factors except essential fatty acids are apparently supplied by rice bran concentrate. Since this material without a supplement of essential fatty acids can limit the dermal lesions to a dry scaliness of the tail and paws (Quackenbush et al., '42), it is more effective than pyridoxine plus pantothenic acid. This fact is taken as evidence for the existence of an additional water-soluble factor. Schneider et al. ('40) reported that an "accessory" factor present in rice bran concentrate was needed to supplement pyridoxine in curing acrodynia. It now becomes evident that their "accessory" factor was multiple in nature, one component being pantothenic acid. Whether the additional factor is a known dietary essential remains to be determined.

### SUMMARY

Rat acrodynia was produced on a diet containing 0.003% of unsaturated fat. Various factors were tested for their curative action. Pantothenic acid did not even alleviate the symptoms. Pyridoxine produced temporary alleviation but did not effect a cure. Ethyl linolate cured the acrodynia. Amounts of linolate which were subcurative alone became curative when given with pyridoxine. Pantothenic acid together with pyridoxine improved the dermal condition, and linolate subsequently produced further improvement. The three compounds together cured the acrodynia but did not cure completely the scaly condition of the tail and hind-paws. The results indicate that an additional factor is involved.

In prophylactic tests neither pantothenic acid nor pyridoxine prevented the acrodynia but pyridoxine retarded the development of the dermal lesions. A lack of pyridoxine did not result in acrodynia when animals were fed both linoleic acid (corn oil) and pantothenic acid.

Sustained growth resulted only when all three supplements were fed.

### LITERATURE CITED

BIRCH, T. W., AND P. GYÖRGY 1936 A study of the chemical nature of vitamin B$_6$ and methods for its preparation in a concentrated state. Biochem. J., vol. 30, p. 304.

BIRCH, T. W., P. GYÖRGY AND L. J. HARRIS 1935 The vitamin B complex. Differentiation of the antiblack-tongue and the ''P.-P.'' factors from lactoflavin and vitamin B$_6$ (so-called ''rat-pellagra'' factor). Parts I–VI. Biochem. J., vol. 29, p. 2847.

BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem., vol. 82, p. 345.

GOLDBERGER, J., AND R. D. LILLIE 1926 A note on an experimental pellagra-like condition in the albino rat. Pub. Health Rep., U. S. P. H. S., vol. 41, p. 1025.

GYÖRGY, P. 1938 Crystalline vitamin B$_6$. J. Am. Chem. Soc., vol. 60, p. 983.

GYÖRGY, P., C. E. POLING AND Y. SUBBAROW 1939 Experiments on the anti-dermatitis component of the filtrate factor in rats. Proc. Soc. Exp. Biol. Med., vol. 42, p. 738.

LEPOVSKY, S. 1938 Crystalline factor 1. Science, vol. 87, p. 169.

QUACKENBUSH, F. W., F. A. KUMMEROW AND H. STEENBOCK 1941 Fat metabolism in rat acrodynia. J. Nutrition, Proc. Am. Inst. Nutrition, vol. 27, p. 11.

———— 1942 Effectiveness of linoleic, arachidonic and linolenic acids in reproduction and lactation. J. Nutrition, vol. 24, p. 213.

QUACKENBUSH, F. W., B. R. PLATZ AND H. STEENBOCK 1939 Rat acrodynia and the essential fatty acids. J. Nutrition, vol. 17, p. 115.

RICHARDSON, L. R., AND A. G. HOGAN 1936 Skin lesions of the rat associated with the vitamin B complex. Mo. Agr. Exp. Sta. Res. Bul., vol. 241.

———— 1941 Factors related to rat dermatitis. J. Biol. Chem., vol. 140, p. cvi.

RICHARDSON, L. R., A. G. HOGAN AND K. F. ITSCHNER 1941 Vitamin B$_6$, pantothenic acid, and unsaturated fatty acids as they affect dermatitis in rats. Research Bulletin, University of Missouri, College of Agriculture, Agricultural Experiment Station, 333. September, pp. 1–12.

SALMON, W. D. 1938 The effect of certain oils in alleviating localized erythematous dermatitis (acrodynia or vitamin B$_6$ deficiency) in rats. J. Biol. Chem., vol. 123, p. civ.

———— 1941 The relation of pantothenic acid, pyridoxine, and linoleic acid to the cure of rat acrodynia. J. Biol. Chem., vol. 140, p. cix.

SCHNEIDER, H., H. STEENBOCK AND B. R. PLATZ 1940 Essential fatty acids, vitamin B$_6$, and other factors in the cure of rat acrodynia. J. Biol. Chem., vol. 132, p. 539.

# NICOTINIC ACID IN FOODS

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Although the clinical use of nicotinic acid has received wide publicity and its physiological action is frequently discussed, yet it is only comparatively recently that the practical problem of its quantitative occurrence has been attacked. One difficulty has been that the present methods of assay are far from being perfect, and frequently give results which are not always capable of substantiation.

It is, therefore, not surprising to find a lack of acceptable average nicotinic acid values such as might be found in tables of food composition. Study of the literature disclosed that, as mentioned above, different methods of assay gave somewhat divergent results. Moreover, marked variations in nicotinic acid values for the same type of item could occur naturally; thus, for example, the previous diet of an animal could play a major role in determining the nicotinic acid content of the animal's tissues. It was, however, necessary to obtain such estimated average values for common foods in so far as our present knowledge would permit. It is in the belief that a résumé of the literature on this subject may prove useful to other workers, that the following table is presented.

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TABLE 1
*Nicotinic acid content — mg./100 gm. fresh weight.*

| FOOD | MICROBIOLOGICAL | | BIOLOGICAL | | CHEMICAL METHOD | |
|---|---|---|---|---|---|---|
| | Value | Ref. | Value | Ref. | Value | References |
| **MEATS — Fresh** | | | | | | |
| *Beef* | | | | | | |
| Brain (4.9) | *15.0 | 31 | 7.5 | 29 | 4.9, 2.4, 3.0, 6.0 | 29, 9, 15, 17 |
| Heart (7.6) | *39.0 | 31 | 4.9 | 29 | 4.9, 5.92, 11.4, 7.6 | 9, 4, 17, 29 |
| Kidney (7.34) | *32.0 | 29 | 16.2 | 29 | 7.34, 4.4, 5.8 | 29, 9, 4 |
| | *26.0 | 31 | | | 10.2, 5.8 | 17, 19 |
| Liver (17.9) | *47.0 | 29 | 26.5 | 29 | 9.2, 17.0, 15.5–20 | 2, 15, 16 |
| | *40.0 | 31 | | | 12.2, 17.7, 17.9 | 4, 17, 29 |
| | | | | | 17.7, 7.7 & 25.0 | 18, 19 |
| | | | | | 8.6, 20.2 | 9, 28 |
| Lung (6.2) | *11.0 | 31 | 8.3 | 29 | 6.2, 6.6 | 29, 17 |
| Muscle (6.39) | *22.0 | 31 | 3.8 | 29 | 4.3, 4.9, 4.9, .02 | 15, 16, 4, 12 |
| | | | 10.2 | 29 | 4.9, 4.8, 6.39, 4.9 | 17, 7, 29, 18 |
| | | | | | 3.5, 4.0, 2.5, 4.8 | 19, 9, 2, 28 |
| Spleen (3.2) | *20.0 | 29 | 9.2 | 29 | 3.2, 6.2, 4.7 | 9, 17, 28 |
| | * 8.9 | 31 | | | | |
| Tongue (7.1) | | | 12.8 | 29 | 6.12, 7.1 | 29, 17 |
| Various cuts | | | | | | |
| Pancreas (5.84) | *15.7 | 29 | 3.2 | 29 | 5.84 | 29 |
| Round | | | | | 7.86 | 29 |
| *Fowl* | | | | | | |
| Chicken (11.2) | | | | | 10.0–13, 11.2–13.5 | 18, 17 |
| | | | | | 4.2 | 9 |
| Dark | | | | | 6.14 | 29 |
| Light (7.3) | *29.0 | 29 | | | 7.3 | 29 |
| Liver (8.0) | | | | | 8.0–9.6, 6.2 | 17, 9 |
| Heart (7.4) | | | | | 7.4, 2.9 | 17, 9 |
| Grouse — fresh | | | | | 6.55 | 18 |
| Liver | | | | | 13.3 | 17 |
| Heart | | | | | 6.9 | 17 |
| *Rabbit* | | | | | | |
| Brain | | | | | 1.2 | 23 |
| Heart | | | | | 3.4 | 23 |
| Liver (14.3) | | | | | 7.85, 14.3 | 23, 29 |
| Lung | | | | | 0.9 | 23 |
| Muscle (6.5) | | | | | 11.0, 6.5 | 29, 23 |
| *Mutton* and *Lamb* | | | | | | |
| Muscle (4.5) | | | | | 4.5, 4.1, 2.8 | 18, 19, 9 |
| Adrenals | | | | | 13.5 | 15 |
| Brain (3.2) | | | | | 2.6, 3.2, 4.7 | 19, 9, 17 |
| Heart (6.0) | | | | | 8.7, 4.4, 6.0, 5.5 | 19, 9, 15 |
| | | | | | | 17 |
| Kidney (6.0) | | | | | 6.0, 7.5, 5.4 | 9, 15, 17 |
| Liver (17.6) | *42.0 | 29 | 42.5 | 29 | 10.1, 20.0 | 9, 15 |
| | | | | | 12.5, 17.6, 12.5 | 17, 29, 18 |
| | | | | | 15.5 & 25.2, 10.1 | 19, 8 |
| Lung | | | | | 3.7 | 17 |
| Pancreas | | | | | 4.0 | 15 |
| Spleen | | | | | 3.8 | 9 |
| Various cuts | | | | | | |
| Leg | | | | | 8.48 | 29 |
| *Pork* | | | | | | |
| Brain (6.4) | *19.0 | 31 | | | 6.4, 10.0 | 9, 17 |
| Heart (7.3) | *24.5 | 31 | 8.0 | 29 | 7.3, 5.34, 7.8 | 9, 4, 17 |
| Kidney (9.8) | *45.5 | 29 | 15.5 | 29 | 9.8, 7.2, 6.82, 7.6 | 29, 9, 4, 17 |
| Liver (22.8) | *53.4 | 24 | | | 28.2, 15.7 | 28, 17 |
| | *54 | 29 | 27.0 | 29 | 22.8, 15.7, 16.0 | 29, 18, 9 |
| | *39 | 31 | | | 11.8 | 4 |
| Lung | *14 | 31 | | | 2.5 | 17 |

' All figures with asterisk on dry basis — others fresh.

TABLE 1 — (*Continued*)

| FOOD | MICROBIOLOGICAL | | BIOLOGICAL | | CHEMICAL METHOD | |
|---|---|---|---|---|---|---|
| | Value | Ref. | Value | Ref. | Value | References |
| *Pork* | | | | | | |
| Muscle | *18.0 | 31 | | | 0.02, 3.1, 5.3 | 12, 17, 28 |
| | | | | | 3.13, 2.6, 6.2, 3.3 | 18, 19, 9, 16 |
| | | | | | 4.73 | 4 |
| Pancreas | | | | | 5.0 | 4 |
| Spleen (4.5) | *12.0 | 31 | | | 5.3, 4.5 | 9, 17 |
| Stomach | | | | | 3.0 | 17 |
| Thymus | | | | | 3.25 | 4 |
| Tongue | | | | | 5.3 | 17 |
| Various cuts | | | | | | |
| Bacon | | | | | 4.4 | 19 |
| Ham (8.8) | | | 8.8 | 29 | 7.14 | 29 |
| Loin (8.8) | *23.5 | 29 | 8.8 | 29 | 6.4 | 29 |
| Smoked ham (8.2) | 14.5 | 29 | 8.2 | 29 | 5.7, 8.3 | 29, 28 |
| Tenderized ham (8.3) | *16.0 | 29 | 8.3 | 29 | 5.8 | 29 |
| *Veal* | | | | | | |
| Heart | | | | | 10.6 | 29 |
| Liver (17.6) | *50 | 24 | 22.5 | 29 | 17.6 | 29 |
| | *41.0 | 29 | | | | |
| Various cuts | | | | | | |
| Hindquarters | | | { 6.5–17.0 } | 29 | 8.4 | 29 |
| *Fish* | | | | | | |
| Cod — Flesh (2.3) | | | | | 1.7, 3.0, 1.7, 1.95 | 18, 15, 16, 4 |
| | | | | | 2.3 | 29 |
| Liver | | | | | 1.6 | 18 |
| Roe (1.52) | | | | | 1.4, 1.52 | 16, 4 |
| Crab (2.8) | | | | | 2.6, 2.82, 2.8 | 19, 18, 17 |
| Cuttle fish | | | | | 0.9 | 19 |
| Flounder | | | | | 3.84 | 18 |
| Halibut | | | | | 6.08 | 18 |
| Herring (3.5) | | | | | 3.5–4.0, 4.0, 2.9 | 18, 15, 16 |
| Milt (2.36) | | | | | 2.36, 2.1 | 18, 15 |
| Roe (2.36) | | | | | 2.36, 2.1 | 18, 15 |
| Ling | | | | | 2.7 | 17 |
| Mackerel (5.5) | | | | | 5.5, 7.2 | 18, 17 |
| Mullet | | | | | 2.9 | 7 |
| Oyster | | | | | 1.3 | 19 |
| Salmon (6.0) | | | | | 6.0, 8.4, 7.35 | 18, 15, 29 |
| *COOKED OR CANNED MEATS AND FISH* | | | | | | |
| *Beef* | | | | | | |
| Heart (7.2) | | | | | 3.3 & 7.2 | 29 |
| Kidney, stewed (5.23) | | | | | 5.23 | 29 |
| Liver, fried (15.8) | *38.0 | 29 | 29.4 | 29 | 15.8 | 29 |
| Round, fried (9.05) | | | | | 9.05, 4.2 | 29, 19 |
| Roasted (4.28) | | | 10.2 | 29 | 4.28 | 29 |
| Boiled 20 min. (2.4) | | | | | 2.4 | 19 |
| Spleen, stewed | *15.8 | 29 | | | 6.3 | 29 |
| *Fowl* | | | | | | |
| Duck, roasted | | | | | 2.8 | 19 |
| Chicken, roasted | | | | | 7.5 | 19 |
| Canned | | | | | 7.4 | 17 |
| Liver, boiled 20 min. (11.2) | | | | | 7.2 & 11.2 | 19 |
| Goose, roasted | | | | | 2.8 | 19 |
| Heart, stewed | | | | | 1.5 | 19 |
| Liver, boiled 20 min. | | | | | 3.6 | 19 |
| *Pork* | | | | | | |
| Brain, boiled 15 min. | | | | | 2.2 | 19 |
| Ham, fried | *14.0 | 29 | | | 3.7 | 29 |
| Boiled (6.3) | *13.0 | 29 | 5.2 | 29 | 6.3 | 29 |

* All figures with asterisk on dry basis — others fresh.

TABLE 1 — *(Continued)*

| FOOD | MICROBIOLOGICAL Value | Ref. | BIOLOGICAL Value | Ref. | CHEMICAL METHOD Value | References |
|---|---|---|---|---|---|---|
| *Pork* | | | | | | |
| Ham, smoked | | | | | | |
|   Fried | *13.9 | 24 | | | 14.3, 1.3 | 29, 19 |
| Kidney, fried | | | | | 4.0 | 19 |
| Liver, fried | | | | | 5.1 & 11.2 | 19 |
| Loin, broil | | | | | 4.45 | 29 |
| Loin, fried | | | | | 5.57 | 29 |
| Sausage, fried | | | | | 1.8 | 19 |
| *Mutton* | | | | | | |
| Boiled, 20 min. | | | | | 3.9 | 19 |
| *Veal* | | | | | | |
| Steak, fried | | | | | 8.38 | 29 |
| *Fish* | | | | | | |
| Brisling sardines in olive oil | | | | | 3.5 | 17 |
| Clam, canned | 1.08 | 32 | | | | |
| Cod, canned | | | | | 1.7 | 16 |
| Cod roe, preserved (1.4) | | | | | 0.9, 1.4 | 17, 16 |
| Herring sardines (2.9) | | | | | 3.35, 2.9 | 18, 16 |
| Kippers, preserved | | | | | 3.4 | 17 |
| Oysters, canned | 0.66 | 32 | | | | |
| Salmon, canned (6.0) | | | | | 6.0, 2.7 | 16, 19 |
| Shrimp | 0.78 | 32 | | | | |
| *EGGS* | | | | | | |
| Hens, white (0.076) | 0.076 | 32 | <2.5 | 30 | <0.05, 1.02, 0.3 | 15, 7, 19 |
| Yolk (0.035) | 0.035 | 32 | <4 | 30 | tr, 1.0, 3.28, tr | 18, 15, 7, 17 |
| Duck, boiled | | | | | 0.2 & 0.3 | 19 |
| *MILK AND PRODUCTS* | | | | | | |
| Cheese, cheddar | | | | | 0.2 | 19 |
| Condensed milk (0.18) | 0.18 | 32 | | | 0.4 | 19 |
| Fresh milk (0.1) | 0.084 | 24 | | | 0.1, 0.05    (0.5) | 19, 17 |
| | 0.08 | 32 | | | 0.054, 0.1, 0.45, (0.1– | 18, 27, 2, 15 |
| | | | | | 0.76, 0.82 | 7, 28 |
| Klim (whole milk) powdered | | | | | 1.1 | 27 |
| Skim milk | | | | | 0.06–.09 | 22 |
| Skim milk, powdered (0.88) | 0.88 | 32 | 4.3–6.2 | 30 | 1.0, 1.4 to 2.8 | 27, 22 |
| | | | 5–15 | 20 | 2.5, 1.2 | 15, 19 |
| *CEREALS AND PRODUCTS* | | | | | | |
| *Bread* | | | | | | |
| White (0.66) | 0.66–0.95 | 32 | | | <0.5, 2.16, 0.2, 4.7 | 15, 6, 19, 7 |
| Brown (1.80) | 1.80 | 32 | | | 1.2, 0.8 | 15, 19 |
| Whole meal (2.88) | 2.88 | 32 | | | 0.7 | 19 |
| *Cereals* | | | | | | |
| Wheat (4.0) | 4.0–4.9 | 32 | | | 3.1, 3.8, 4.7–5.3, 3.9 | 8, 27, 1, 7 |
| Oats (1.1) | 1.1 | 32 | | | 2.6–1.0 | 19, 1 |
| Corn, yellow (dry whole) | | | | | | |
|   (1.56) | 2.1 | 24 | | | 1.1, 1.3 | 7, 18 |
| | 1.56–2.6 | 32 | | | 0.2–0.8, 1.4–2.2 | 8, 19 |
| | | | | | 1.5, 1.4, 1.3 | 27, 1, 16 |
| Corn, white (1.0) | | | | | 1.3–1.6, 1.3, 1.0 | 27, 1, 7 |
| | | | | | 1.6, 0.7–1.5 | 1, 25 |
| | | | | | 4.4, 2.8, 5.6 | 8, 7, 9 |
| Wheat flour, white (0.9) | 5.2 | 24 | | | .81 | 6 |
| | 0.9–1.1 | 32 | | | 1.3, 0.9–1.1, 4.4–3.8 | 19, 1, 7 |
| Sorgum (*sorgum vulgare*) | | | | | 2.2, 2.1 | 8, 25 |
| Buckwheat | 4.4 | 32 | | | | |
| Rye flour, whole (1.22) | 6.3 | 24 | | | | |
| | 1.22 | 10 | | | 1.3 | 16 |
| Rye flour, white unbleached | 0.71 | 10 | | | | |
| Rye flour, white bleached | 0.73 | 10 | | | | |

* All figures with asterisk on dry basis — others fresh.

TABLE 1 — *(Continued)*

| FOOD | MICROBIOLOGICAL | | BIOLOGICAL | | CHEMICAL METHOD | |
|---|---|---|---|---|---|---|
| | Value | Ref. | Value | Ref. | Value | References |
| *CEREALS AND PRODUCTS* | | | | | | |
| Rye flour, degerminated | | | | | | |
|   bleached | 0.73 | 10 | | | | |
|   Rice, flour | | | | | 2.4 | 1 |
|     White, polished (0.90) | 0.90 | 32 | | | 0.9, 1.5, 3.1 | 19, 27, 7 |
|     Parboiled, then polished | | | | | | |
|      (3.0) | | | | | 2.6, 3.0, 3.8 | 19, 27, 1 |
|   Rice, unpolished (6.0) | | | | | 6.0–6.4 | 19 |
|     Milled (1.6) | | | | | 1.7, 2.1–3.2, 1.6 | 26, 8, 1 |
|   Millet (0.8) | | | | | 0.8, trace | 27, 8 |
|   Rye (1.1) | 0.9–1.3 | 32 | | | | |
|   Barley (4.7) | 4.7 | 32 | | | 2.5–3.0 | 1 |
|   Flour | 5.7 | 24 | | | | |
|   Oatmeal (0.6) | 1.0–1.5 | 24 | | | 0.6 | 19 |
|   Pearl Barley (2.7) | 2.7 | 32 | | | 3.5 | 19 |
|   Corn meal, yellow (0.6) | 0.6–1.0 | 24 | | | | |
| | 0.6 | 24 | | | 0.6–0.8, 1.0 | 15, 7 |
| | 1.0 | 32 | | | | |
|     White (0.9) | 1.76 | 32 | | | 0.9 | 1 |
|     Flour | | | | | 0.3 | 1 |
|   Spaghetti (2.1) | 2.1 | 32 | | | 3.28 | 7 |
|   Tapioca — boiled 20 min. | | | | | 0.3 | 19 |
| *BEANS AND OTHERS, DRIED* | | | | | | |
|   Kidney bean (2.8) | 2.8 | 32 | | | 2.8 | 7 |
|     (*phasoleus vulgaris*) | | | | | | |
|   Lima beans | 1.83 | 32 | | | | |
|   Broad bean | | | | | 2.1 | 7 |
|   Soy bean (4.32) | | | | | 1.2, 4.32, 4.25 | 19, 3, 25 |
|   Mung bean (*phasoleus aureus*) | | | | | 2.3 | 8 |
|   Lentils | | | | | 3.1 | 7 |
|   Peas (1.8) | | | | | 2.0, 1.0, 1.8 | 7, 1, 1 |
|     chick (*cicer arientinum*) | | | | | 3.89 | 7 |
|   Peanuts, raw | | | | | 5.9 | 19 |
|   Peanut butter, 6% fat | | | | | 20–23 | 2 |
|     Norm. fat | 18.6 | 32 | | | | |
|   Meal extracted (17.2) | 17.2 | 24 | 13 | 30 | 16.7, 22 | 25, 2 |
|   Coconut, raw | | | | | 0.4 | 19 |
|   Nuts, (unspecified type) | | | | | 1.29 | 7 |
|     Almonds | | | | | 1.82 | 7 |
|     Chestnuts | | | | | 1.17 | 7 |
| *VEGETABLES — LEAFY, GREEN AND YELLOW* | | | | | | |
|   Bamboo shoots | | | | | 0.2 | 19 |
|   Beans, green (0.64) | 0.64 | 32 | | | 0.1, 0.3 | 19, 19 |
|   Broccoli, leaves | | | | | 1.44 | 7 |
|     Stem (lower) | | | | | 0.97 | 7 |
|   Cabbage (0.29) | 0.29 | 32 | | | 0.4, 0.3 | 19, 15 |
|   Carrots (1.47) | 1.47 | 32 | | | <0.5 | 15 |
|   Endive | | | | | .72 | 7 |
|   Kohlrabi | 0.27 | 32 | | | | - |
|   Lettuce | | | | | 0.5 | 19 |
|   Lima beans, green (0.29) | 0.29 | 32 | | | 0.8 | 19 |
|   Mangels, leaves (0.3) | | | | | 0.3 | 17 |
|     Stems (0.13) | | | | | 0.13 | 17 |
|   Peas, green | | | | | 0.7 | 19 |
|   Peppers, green (0.2) | | | | | 0.2, .76 | 19, 7 |
|   Pumpkin (0.7) | | | | | 0.7 | 19 |
|   Spinach (0.72) | 0.72 | 32 | | | 1.7 | 15 |
|   Squash, Zucchini (*Curcubita lagenaria*) | | | | | 0.96 | 7 |
| *VEGETABLES, LEAFY, GREEN AND YELLOW, canned* | | | | | | |
|   Asparagus bean | | | | | 0.5 | 17 |
|   Beans, wax | | | | | 0.6 | 17 |
|     French | | | | | 0.05 | 17 |
|   Peas | | | | | 1.3, 0.3 | 17, 19 |
|   Spinach | | | | | 0.7 | 17 |

* All figures with asterisk on dry basis — others fresh.

TABLE 1 — (*Continued*)

| FOOD | MICROBIOLOGICAL | | BIOLOGICAL | | CHEMICAL METHOD | |
|------|-------|------|-------|------|-------|-----------|
| | Value | Ref. | Value | Ref. | Value | References |
| **VEGETABLES**, *other than leafy green or yellow, fresh* | | | | | | |
| Cucumber (0.32) | 0.32 | 32 | | | <0.1 | 19 |
| Cauliflower | 0.57 | 32 | | | | |
| Eggplant (0.6) | | | | | 0.6, 0.86 | 19, 7 |
| Gourd | | | | | 0.1 | 19 |
| Onion (0.10) | 0.10 | 32 | | | <0.1 | 19 |
| Radish | | | | | <0.1 | 19 |
| **VEGETABLES**, *other, canned* | | | | | | |
| Beets, red (0.64) | 0.64 | 32 | | | 0.3 | 17 |
| **POTATOES** | | | | | | |
| White (1.18) | 1.18 | 32 | | | 0.4, 2.0, 1.0, 1.07 | 19, 15, 16, 7 |
| Sweet (1.29) | 1.29 | 32 | | | <0.1 | 19 |
| Yam (0.67) | 0.67 | 32 | | | | |
| **TOMATOES** | | | | | | |
| Fresh (0.58) | 0.58 | 32 | | | 0.1, <0.5, .99 | 19, 15, 17 |
| **FRUITS**, *other than citrus* | | | | | | |
| Apples (0.50) | 0.50 | 32 | | | 0.2, 0.5, 1.29 | 19, 15, 7 |
| Banana | 0.61 | 32 | | | | |
| Berries (no type given) | | | | | 1.63 | 7 |
| Cranberries | 1.29 | 32 | | | | |
| Dates | 2.18 | 32 | | | | |
| Figs, fresh | | | | | 0.63 | 7 |
| Grapes, pulp | | | | | 0.84 | 7 |
| Peel | | | | | 0.94 | 7 |
| Guava | | | | | 1.1 | 19 |
| Peaches | 0.95 | 32 | | | | |
| Pear | 0.14 | 32 | | | | |
| Plum | 0.56 | 32 | | | | |
| Watermelon | | | | | <0.1 | 19 |
| **FRUITS**, *dried* | | | | | | |
| Figs | | | | | 1.72 | 7 |
| Raisins | 0.63 | 32 | | | | |
| **MISCELLANEOUS** | | | | | | |
| Yeast, bakers, fresh (11.0) | | | | | 11.0, 9.9, 11.0 | 16, 7, 17 |
| Yeast, dried (40) | 40–50 | 32 | 50 | 30 | 29–32, 25.7, 15 & 26 | 2, 4, 19 |
| Yeast, brewers, dried (40) | 40–60 | 32 | 34–93 | 30 | 35.5 | 18 |
| | | | | | 60.6, 48.3, 41 | 8, 27, 2 |
| | | | | | 44, 25–45 | 16, 17 |
| Yeast, brewers, fresh (10.2) | | | | | 10.2, 9.1 | 16, 15 |
| **CONCENTRATES** — *etc.* | | | | | | |
| Beef extract | | | | | 20.4 | 19 |
| Rice bran (polishings) | | | | | 30.0 | 27 |
| Bran concentrates | | | | | 140 | 17 |
| Wheat germ (4.2) | | | <4 | 30 | 2.7, 4.2 | 15, 16 |
| Germ extracted | | | <6 | 30 | | |
| Bran (4.2) | | | | | 5.0, 5.1, 4.2 | 16, 7, 16 |

' All figures with asterisk on dry basis — others fresh.

The foods have been grouped by classes according to similar nutritive content or unique contribution to the diet. Such a classification is useful because it not only makes possible a rapid and reasonably accurate evaluation of a diet, but also does away with the tedious procedure of evaluating food items singly, even though they are of similar nutritive value. The classes of foods on this basis are as follows: meats; eggs; milk and milk products; butter; other fats; grains and grain products; legumes; sugars and syrups; leafy, green or yellow vegetables; tomatoes; citrus fruits; potatoes; vegetables other than leafy, green or yellow; fruits other than citrus; dried fruits; beverages; and miscellaneous.

The values for each food as found in the literature have been arranged in columns titled according to the method of assay. Adjacent columns termed "Reference" pertain to the source of information as indicated in the numbered bibliography. Where several values have been found in the literature, we have indicated in parentheses (adjacent to the name of the item) what is believed to be a reasonably accurate average value. Usually, the values so chosen represent those obtained by North American workers on native material. It must be emphasized, however, that these decisions will bear constant revision in the light of future findings, and that they do not in any way represent a final opinion.

### LITERATURE CITED

1. AYKROYD, W. R., AND M. SWAMINATHAN 1940 The nicotinic acid content of cereals and pellagra. Ind. J. Med. Res., vol. 27, p. 667.

2. ARNOLD, AARON, C. B. SCHREFFLER AND A. T. LIPSIUS 1941 Chemical determination of nicotinic acid. Ind. and Eng. Chem., Anal. Ed., vol. 13, p. 61.

3. ASHFORD, WALTER R., AND R. H. CLARK 1939 A comparison of colorimetric methods for the quantitative determination of nicotinic acid. Trans. Royal Soc. Canada, 3rd ser. 33, Sec. 3, p. 29.

4. BANDIER, ERIK 1939 Quantitative estimation of nicotinic acid in biological material. Biochem. J., vol. 33, p. 1130.

5. BANDIER, E., AND J. HALD 1939 A colorimetric reaction for the quantitative estimation of nicotinic acid. Biochem. J., vol. 33, p. 264.

6. BINA, ALBERT F., JAMES THOMAS AND ELMER B. BROWN 1941 The determination of nicotinic acid in bread and other cereal products. Cereal Chemistry, vol. 18, p. 661.

7. DELREGNA, DI F. 1939 The nicotinic acid content of food materials. Quaderni Nutriz., vol. 6, p. 368.

8. GIRI, K. V., AND B. NAGANNA 1941 An adoption method for the estimation of the nicotinic acid content of foodstuffs. Ind. J. Med. Res., vol. 29, p. 125.

9. GIRI, K. V., AND B. NAGANNA 1941 Estimation of nicotinic acid in tissue and blood. Ind. J. Med. Res., vol. 29, p. 585.

10. IDHE, AARON J., AND HENRY A. SCHUETTE 1941 Thiamin, nicotinic acid, riboflavin, and pantothenic acid in rye and its milled products. J. Nutrition, vol. 22, p. 527.

11. KARRER, P., AND H. KELLER 1938 A colorimetric determination of nicotinic acid amide. Helv. Chim. Acta., vol. 21, p. 463.

12. KARRER, P., AND H. KELLER 1938 Quantitative determination of nicotinic acid amide in animal organs. Helv. Chim. Acta., vol. 21, p. 1170.

13. KARRER, P., AND H. KELLER 1939 Determination of nicotinic acid in animal organs. Helv. Chim. Acta., vol. 22, p. 1292.

14. KODICEK, E. 1940 Estimation of nicotinic acid in animal tissues, blood, and certain foodstuffs. I. Method. Biochem. J., vol. 34, p. 712.

15. KODICEK, E. 1940 Estimation of nicotinic acid. Biochem. J., vol. 34, p. 724.

16. KRINGSTAD, H., AND T. NAESS 1939 A colorimetric method for the determination of nicotinic acid and nicotinic acid amide in foodstuffs. Z. Physiol. Chem., vol. 260, p. 108.

17. KRINGSTAD, HANS, AND FINN THORENSEN 1940 The occurrence of the antipellagra vitamin, nicotinic acid (amide) in some foods. Nord. Med., vol. 48, p. 2248.

18. KRINGSTAD, H., AND F. THORENSEN 1940 The occurrence of the antipellagra vitamin (nicotinic acid, nicotinamide) in fish and fish products. Tids. Hermetikind., vol. 26, p. 114.

19. LEONG, P. C. 1940 Nicotinic acid content in foods. J. of the Malaya Branch of the Brit. Med. Assn., vol. 4, p. 261.

20. McKIBBIN, J. M., HARRY WAISMAN, O. MICKELSON AND C. A. ELVEHJEM 1939 Bull. Wisc. Agric. Exp. Sta., vol. 446, p. 40.

21. MELNICK, DANIEL, AND HENRY FIELD 1940 Determination of nicotinic acid in biological materials by means of photoelectric colorimetry. J. Biol. Chem., vol. 134, p. 1.

22. NOLL, C. I., AND O. G. JENSEN 1941 The chemical determination of nicotinic acid in milk and milk derivatives. J. Biol. Chem., vol. 140, p. 755.

23. RITSERT, K. 1939 On the quantitative nicotinic acid amide content of urine, tissue, and blood. Klin. Wochenschr., vol. 18, p. 934.

24. SNELL, E. E., AND L. D. WRIGHT 1941 A microbiological method for the determination of nicotinic acid. J. Biol. Chem., vol. 139, p. 675.

25. SWAMINATHAN, M. 1938 A colorimetric method for the determination of nicotinic acid in foodstuffs. Ind. J. Med. Res., vol. 26, p. 427.

26. SWAMINATHAN, M. 1941 The effect of washing and cooking on the nicotinic acid content of raw and parboiled rice. Ind. J. Med. Res., vol. 29, p. 83.

27. SWAMINATHAN, M. 1941 Cyanogen bromide method of estimating nicotinic acid. Ind. J. Med. Res., vol. 29, p. 325.

28. WAISMAN, HARRY A., AND C. A. ELVEHJEM 1941 Chemical estimation of nicotinic acid and vitamin B₆. Ind. and Eng. Chem., Anal. Ed., vol. 13, p. 221.

29. WAISMAN, HARRY A., AND C. A. ELVEHJEM 1941 The vitamin content of meat. Burgess, Minneapolis.

30. WAISMAN, HARRY A., OLAF MICKELSEN, J. M. McKIBBIN AND C. A. ELVEHJEM 1940 Nicotinic acid potency of food materials and certain chemical compounds. J. Nutrition, vol. 19, p. 483.

31. WILLIAMS, R. J., AND OTHERS 1941 Studies on the vitamin content of tissues. The University of Texas Publication, vol. 4137, pp. 1–87.

32. TEPLY, E. J., F. M. STRONG AND C. A. ELVEHJEM 1942 The distribution of nicotinic acid in foods. J. Nutrition, vol. 23, p. 417.

33. BACHARACH, A. L. 1941 The distribution of nicotinic acid in human and animal foods. Nutr. Abs. and Reviews, vol. 10, p. 459.

# A SALT MIXTURE FOR USE WITH BASAL DIETS EITHER LOW OR HIGH IN PHOSPHORUS

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Numerous salt mixtures have been used in the past to supply the necessary inorganic elements in synthetic diets used for experimental purposes. These mixtures have varied considerably in composition but usually they were used with basal diets containing casein and yeast. Both of these materials are rather high in phosphorus and when fed at the usual levels of approximately 18% and 6%, respectively, furnish appreciable amounts of this element. Recent advances in nutrition have made possible the development of experimental diets of an increasingly high degree of purity. In diets containing neither yeast nor casein, or other phosphoprotein, there is no appreciable source of phosphorus except that added as salt.

The possibility that diets low in phosphorus will be used with increasing frequency makes it necessary to have available a salt mixture which will be satisfactory under these conditions. It is also desirable to have a single salt mixture for general laboratory use which will give good results when used with basal diets either high or low in phosphorus. The construction of such a mixture would appear possible from the work of Bethke, Kick and Wilder ('32). These authors have reported good calcification on diets in which the absolute and relative amounts of calcium and phosphorus have been varied within fairly wide limits. In our work with purified

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diets low in phosphorus it was necessary to have a salt mixture which would assure an ample supply of all the inorganic elements and not only produce good growth and well-calcified bones, but also allow for reproduction and lactation. Consequently, after a study of the literature concerning the needs of the rat for the various inorganic elements we constructed a salt mixture which appeared to supply these elements in amounts as near the optimum as possible. In constructing a salt mixture the following are desirable qualifications: (a) the mixture should furnish all the inorganic elements known to be indispensable for mammals and in approximately the relative amounts demanded; (b) the minimum number of salts should be used; (c) all of the salts should be inorganic; (d) the mixture should be easily prepared; (e) the base-forming elements should be only slightly in excess of the acid-forming elements; and (f) finally the exact amount of each salt chosen should be expressed in moles or a fraction of a mole to facilitate equimolecular substitution when desired.

A few years ago Hubbell, Mendel and Wakeman ('37) reported the results of using a salt mixture (salts no. 351) which is high in calcium and low in phosphorus. This mixture, which is composed of over 50% calcium carbonate, was employed by the above authors with a basal diet containing casein and a high level (8%) of yeast. Very good results in respect to both growth and bone formation were obtained when the total salt intake was only 2% of the diet, but for ordinary feeding experiments 2.5% was recommended. Hubbell and associates also used this salt mixture with diets containing lactalbumin as the protein "to determine what type of growth and what sort of bone would be produced when the only source of phosphorus was that found in the salt mixture itself." They suggested that under these conditions the total salt intake be increased to 3%. The albumin diet, however, still contained 8% of yeast. This quantity of yeast would supply approximately 0.13% phosphorus or about half the total phosphorus in the basal casein-containing diet and nearly as much as that present in the salt mixture if the latter

were fed at 3%. The results obtained with this amount of phosphorus in the basal diet cannot be interpreted as showing what might take place if the basal diet were really devoid of phosphorus and "the only source of phosphorus was that found in the salt mixture itself."

The total phosphorus of the casein-yeast diet after the addition of 2% of the salt mixture was approximately 0.345%. In order to supply this amount, if the salt mixture was the only source of phosphorus, it would be necessary to feed over 6 gm. of the salt mixture per 100 gm. of diet, and the calcium to phosphorus ratio would be greater than 4 to 1. This salt mixture has been used by other investigators rather widely, and little attention has been given to the amount of phosphorus in the basal diet. Sure [1] ('40), for example, has used this salt mixture at a 4% level with a diet containing fibrin as a protein, and the B factors, for the most part, furnished as pure substances. Such a basal diet is very low in phosphorus.

We chose salts no. 351 to compare with our salt mixture (salts no. 12) for the following reasons: (a) with the basal diet they used, Hubbell et al. found that salts no. 351 produced very good growth and calcification; (b) salt mixture 351 has been very widely used by others; and (c) because of the high calcium to phosphorus ratio it did not seem that salts no. 351 would be a satisfactory mixture to use with a basal diet low in phosphorus. Salt mixture 12 has been compared with salts no. 351 in its ability both to produce growth and calcification of bone and to maintain normal concentrations of serum calcium and inorganic phosphorus. The salt mixtures were compared at various levels using two different basal diets — one low in phosphorus and the other moderately high in phosphorus. The composition of salt mixture 12 and the results of comparing it with salts no. 351 are given below.

## EXPERIMENTAL

Salt mixture 12, the composition of which is given in table 1, is quite similar to salts no. I of Phillips and Hart ('35), but

[1] Sure ('41) has recently reported poor reproduction and lactation with salt mixture 351.

the latter does not fulfill all the qualifications previously listed. Furthermore, salt mixture 12 contains more manganese [2] and a trace of cobalt, and the phosphate is supplied as the monopotassium instead of the dipotassium salt. The use of the monobasic phosphate decreases the amount of potassium and makes the salt mixture more nearly neutral. This mixture contains neither aluminum nor fluorine. There is little evidence that animals require aluminum, and Evans and Phillips

TABLE 1

*Composition of salt mixture 12.*

| SALT | MOLS | GRAMS | ELEMENT | AMOUNT OF ELEMENT IN 4 GM. OF SALT MIXTURE [1] |
|---|---|---|---|---|
| NaCl | 5 | 292.5 | Na | 0.206 |
| KH$_2$PO$_4$ | 6 | 816.6 | Cl | 0.319 |
| MgSO$_4$ | 1 | 120.3 | K | 0.421 |
| CaCO$_3$ | 8 | 800.8 | P | 0.334 |
| FeSO$_4$·7H$_2$O | 0.2 | 56.6 | Mg | 0.043 |
| KI | 0.01 | 1.66 | Ca | 0.576 |
| MnSO$_4$·2H$_2$O | 0.05 | 9.35 | Fe | 0.020 |
| ZnCl$_2$ | 0.004 | 0.5452 | I | 0.00228 |
| CuSO$_4$·5H$_2$O | 0.004 | 0.9988 | Mn | 0.00494 |
| CoCl$_2$·6H$_2$O | 0.0002 | 0.0476 | Zn | 0.00047 |
| | | | Cu | 0.00046 |
| | | | Co | 0.00002 |

[1] Equivalent to amount added to 100 gm. of diet if salt mixture is fed at 4%. Calculated on a dry basis.

('39) have shown that if fluorine is necessary the amount needed is so small that they were unable to demonstrate its essential nature. If thought desirable, a small amount of fluoride could be added without materially disturbing the composition of the salt mixture with respect to the other elements. The calcium to phosphorus ratio of this mixture is 1.7 to 1. Hubbell et al. obtained maximum calcification on their casein-

[2] Daniels and Everson ('35) and Richardson and Hogan ('40) have pointed out that the demand for manganese is considerably increased during reproduction and lactation. The chick also requires comparatively large amounts of manganese, and since salt mixture 12 was first constructed and used, Waisman, Mickelsen, McKibbin and Elvehjem ('40) have modified salts no. I of Phillips and Hart to contain more manganese when using it with the chick.

yeast diet with 2% of salts no. 351. This diet contained a total of 0.473% calcium and 0.347% phosphorus. Salt mixture 12 was so constructed that 4% of the mixture would supply approximately this amount of phosphorus (0.334%) and slightly more calcium (0.576%).

All the salts used in the preparation of the mixture for the following studies were of either reagent or c.p. quality. The salts which are present in small quantities were weighed on an analytical balance and, with the exception of the iodide, were ground together in a mortar and worked into the sodium chloride. Free iodine was liberated if the iodide was included with these salts, so it was worked into the sodium chloride separately. Due to the excess of basic over acidic radicals the tendency to lose iodine from the final mixture is reduced, but in cases where a very high degree of purity is not required a stabilized form of iodide such as that suggested by Johnson and Frederick ('40) could be used. The final salt mixture was ground and passed through a 40-mesh sieve.

The low-phosphorus basal diet [3] used for the comparison of the two salts (salts no. 12 and salts no. 351) was composed of the following ingredients expressed in per cent: Alcohol extracted fibrin 18, lard 10, dextrinized starch 67, agar 2, liver extract 2 [4] and glucose [5] containing 20 mg. of thiamine chloride [6] per 100 gm., 1. Two-tenths milliliter of cod liver oil was given to each animal from a tuberculin syringe three times per week. This diet contains from 0.02 to 0.03% of both calcium and phosphorus. Each salt mixture was fed at three levels — 2, 3 or 4 gm. per 100 gm. of basal diet.

Rats were used as experimental animals. Shortly after birth, usually on the second day, the litter of young rats was reduced to six. Only those litters were used in which it was possible to have all six remaining animals of the same sex. At 25 days of age the animals were placed on experiment.

[3] Based on previous reports from this laboratory (Jones, '38, '39).
[4] Liver fraction E supplied by Dr. C. E. Graham of The Wilson Laboratories, Chicago, Illinois.
[5] Cerelose.
[6] Supplied by Merck and Co., Inc., Rahway, N. J.

A total of sixteen litters was used for this comparison and the litter-mates were distributed so that each dietary group included one animal of each litter. This gave identical distribution among the various groups of animals in regard to litters and sex. Without exception the experimental diets were given for 28 days.

At the conclusion of the feeding period the animals of some of the litters were bled under ether anesthesia, and the sera analyzed for calcium by the method of Clark and Collip ('25) and for inorganic phosphorus by the method of Gunther and Greenberg ('29). The remaining animals were chloroformed. The right femurs of all animals were removed and the percentage ash determined on the lipid-free bones. The results of the determinations on the sera and femurs are given as averages for each group in the first section of table 2. Both the absolute and relative amounts of femur ash are recorded. In addition the initial body weight and gain in weight for each group are given.

From the data given in table 2 it is clear that at all levels salt mixture 12 was more effective in producing growth and calcification and in maintaining normal amounts of calcium and inorganic phosphorus in the serum than was salt mixture 351 at the corresponding level. Not only is there a definite difference in the averages when one mixture is compared with the other at the same level, but judged by either the absolute or relative amounts of femur ash, there was not a single animal receiving salts no. 351 which showed as much calcification as its litter-mate receiving an equal amount of salts no. 12. With two exceptions the same was true for gain in body weight. The calcium of the serum was higher and the inorganic phosphorus lower at all levels of salts no. 351 than at any level of salts no. 12, reflecting the difference in ratio as well as absolute amounts of calcium and phosphorus in the salt mixture. With salts no. 351 the calcium was definitely above and the inorganic phosphorus definitely below normal.

TABLE 2

The effect of various salt mixtures on calcification and concentrations of calcium and phosphorus in blood serum.

| GROUP | SALT MIXTURE | AMOUNT OF SALT MIXTURE | INITIAL WEIGHT OF ANIMALS | GAIN IN WEIGHT OF ANIMALS | ASH OF FEMURS | | Number of animals | Number of determinations | SERUM | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Ca | P |
| no. | no. | % | gm. | gm.[1] | mg.[1] | %[1] | | | mg./100 cc.[1] | mg./100 cc.[1] |
| I. Comparison of salt mixtures 351 and 12 on a basal diet low in phosphorus | | | | | | | | | | |
| | | | | | | | | | | 16 animals in each group |
| 1 | 351 | 2 | 50.6 | 58.5 ± 6.1 | 53.7 ± 3.2 | 38.7 ± 4.5 | 8 | 6 | 14.66 ± 0.19 | 6.87 ± 0.35 |
| 2 | 12 | 2 | 50.7 | 94.1 ± 5.4 | 99.3 ± 4.4 | 49.8 ± 0.79 | 6 | 5 | 12.60 ± 0.13 | 8.11 ± 0.53 |
| 3 | 351 | 3 | 50.8 | 68.3 ± 3.9 | 78.4 ± 4.1 | 46.0 ± 0.91 | 8 | 6 | 14.20 ± 0.33 | 7.42 ± 0.55 |
| 4 | 12 | 3 | 50.7 | 100.4 ± 4.4 | 130.2 ± 7.6 | 54.4 ± 0.26 | 8 | 6 | 12.30 ± 0.22 | 9.15 ± 0.45 |
| 5 | 351 | 4 | 50.7 | 75.9 ± 6.5 | 98.1 ± 3.9 | 49.9 ± 0.70 | 8 | 6 | 13.96 ± 0.36 | 7.45 ± 0.29 |
| 6 | 12 | 4 | 50.7 | 102.1 ± 2.4 | 138.1 ± 2.9 | 55.7 ± 0.41 | 8 | 6 | 12.36 ± 0.14 | 9.19 ± 0.38 |
| II. Comparison of salt mixtures 351 and 12 on a basal diet high in phosphorus | | | | | | | | | | |
| | | | | | | | | | | 14 animals in each group |
| 7 | 351 | 2 | 53.8 | 123.2 ± 4.4 | 146.5 ± 2.3 | 55.3 ± 0.40 [2] | 7 | 7 | 11.99 ± 0.19 | 9.85 ± 0.47 |
| 8 | 12 | 2 | 53.9 | 118.4 ± 5.9 | 132.4 ± 2.3 [2] | 54.1 ± 0.40 [2] | 7 | 7 | 11.30 ± 0.23 | 10.30 ± 0.22 |
| 9 | 351 | 3 | 53.9 | 121.3 ± 6.2 | 147.8 ± 4.8 | 55.2 ± 0.61 [2] | 7 | 7 | 12.23 ± 0.22 | 7.92 ± 0.19 |
| 10 | 12 | 3 | 54.5 | 124.8 ± 6.4 [2] | 146.3 ± 3.6 [2] | 55.6 ± 1.2 [2] | 7 | 7 | 11.61 ± 0.16 | 10.24 ± 0.33 |
| 11 | 351 | 4 | 53.8 | 117.0 ± 5.3 | 148.2 ± 3.2 | 55.3 ± 0.45 | 7 | 7 | 13.01 ± 0.23 | 8.59 ± 0.55 |
| 12 | 12 | 4 | 53.9 | 121.0 ± 6.2 | 147.9 ± 4.2 | 55.7 ± 0.48 | 7 | 7 | 12.09 ± 0.19 | 10.12 ± 0.39 |
| III. Comparison of salt mixtures 12, 14 and 14 + 0.25% phosphorus on a basal diet low in phosphorus | | | | | | | | | | |
| | | | | | | | | | | 12 animals in each group |
| 13 | 12 | 4 | 52.1 | 89.5 ± 2.5 | 135.4 ± 4.2 | 55.3[2] ± 0.46 | 8 | 7 | 12.26 ± 0.23 | 8.38 ± 0.35 |
| 14 | 14 | 4 | 52.1 | 89.6 ± 5.2 | 133.4 ± 3.3 | 54.4 ± 0.37 | 8 | 7 | 11.67 ± 0.38 | 8.51 ± 0.58 |
| 15 | 14 + 0.25% P | 4 | 52.1 | 82.0 ± 5.0 | 122.3 ± 2.3 | 54.3 ± 0.41 | 8 | 7 | 12.13 ± 0.11 | 9.08 ± 0.34 |

[1] Standard error of the mean follows the average.
[2] Average of thirteen animals.
[3] Average of twelve animals.

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It is clear from the above results that salt mixture 351 is not satisfactory for use with a basal diet containing negligible amounts of phosphorus even when the mixture is fed in amounts of 4 gm. per 100 gm. of basal diet. On the other hand, the feeding of a salt mixture of higher phosphorus and lower calcium content in amounts of either 3 or 4 gm. per 100 gm. of the same basal diet produces much better calcification and allows for greater growth. These results are in agreement with those of Shohl ('36) who showed that a diet containing calcium and phosphorus in the amounts furnished by 2 to 4 gm. of salts no. 351 per 100 gm. of basal diet and no other source of phosphorus would be definitely rachitogenic for the rat.

After finding that salt mixture 12 was more satisfactory than salts no. 351 for use with a basal diet low in phosphorus, the two mixtures were then compared with each other when used with a basal diet containing liberal amounts of phosphorus. For this purpose the following diet expressed in per cent was used: crude casein 18,[7] dried brewer's yeast 6,[8] agar 2, lard 10, and dextrinized starch 64. Cod liver oil was given as above. The two salt mixtures were again fed at levels of 2, 3 and 4 gm. per 100 gm. of the basal diet. The same technique was followed with regard to choice and distribution of animals and experimental procedure. Fourteen litters of animals were used and calcium and phosphorus were determined on the sera of seven individual animals from each group.

The results of this comparison are given in the second part of table 2. Judged by any of the criteria, there is very little difference between the two salt mixtures. At the 2-gm. level, salts no. 351 appears to be a little superior to salts no. 12 both in producing calcification and growth. At the other levels the two mixtures are about equal. Although not very great, there is a constant difference in serum calcium and phosphorus. As in the first series of experiments, calcium was

greater and the inorganic phosphorus was less in those animals receiving salts no. 351 than in those given salt mixture 12. There was less variation in the degree of calcification among the several groups in the latter, than in the first, series of experiments. This was particularly true with salts no. 351 which emphasizes the necessity of having a liberal amount of phosphorus in the basal diet if this mixture is to be used successfully.

The animals receiving the high-phosphorus basal diet grew more with either salt mixture and at all levels than any of the groups of animals receiving the low-phosphorus basal diet. This might be interpreted as indicating that even with salts no. 12 at the 4-gm. level there was still a deficiency of phosphorus. In view of this difference a direct comparison was made between the low-phosphorus (fibrin and liver extract) diet and the high-phosphorus (casein and yeast) diet with 4 gm. of salt mixture 12 added to 100 gm. of each diet. Seven animals comprised each group. Here again growth was definitely better on the diet containing the higher amount of phosphorus, but there was little difference in calcification.

In another series of experiments the effect of adding phosphorus as monopotassium phosphate to the low-phosphorus diet was studied. The extra phosphorus increased calcification when only 2 gm. of salts 12 per 100 gm. of basal diet were fed, but not when the larger amounts of the salt mixture were given.

As a final experiment, salts no. 12 were compared with salt mixture no. 14. The latter mixture is the same as salts no. 12 except that 4 moles of the calcium carbonate were replaced with an equal number of moles of dicalcium phosphate ($CaHPO_4$). This increases the phosphorus considerably without appreciably altering the relative amounts of the other constituents. When fed at a level of 4% of the diet, salt mixture 14 supplies 0.57% calcium and 0.55% phosphorus with a calcium to phosphorus ratio of 1.04. When a high phosphorus mixture like salts no. 14 is used with a high-phosphorus basal diet such as the casein-yeast diets, there may be danger of

supplying too much phosphorus in comparison with the calcium. Therefore, in addition to comparing salt mixtures 12 and 14 at the 4-gm. level, another group of animals was given the same amount of salts no. 14 plus an additional 0.25% phosphorus as disodium phosphate. This additional phosphorus is approximately the amount supplied by 18% of casein and 6% of yeast which are so frequently used in synthetic diets. The calcium to phosphorus ratio of such a diet is 0.71. The results of this experiment are summarized in the third section of table 2. Here again growth was not so good as on the casein-yeast diet, but in this experiment there was a larger proportion of females than in any of the other series. It does, however, seem that regardless of the amount of phosphorus added, growth on the low-phosphorus basal diet was somewhat below that on the high-phosphorus diet. This is probably due to an insufficiency of some members of the vitamin B complex. The levels of serum calcium and inorganic phosphorus are about the same in all three groups and there is practically no difference in the percentage of femur ash. Growth on the diet containing the largest amount of phosphorus was slightly below that of the other two groups, as was also the weight of femur ash. It seems that this high amount of phosphorus and rather low calcium to phosphorus ratio may have slightly inhibited growth and calcification. If this is the case salt mixture 14 would not be so satisfactory as salts no. 12 when used with a basal diet containing a liberal amount of phosphorus, but salt mixture 12 appears to produce equally good results as salts no. 14 when used with a basal diet low in phosphorus. It seems safe to conclude that 4 gm. of salt mixture 12 per 100 gm. of basal diet and in the presence of ample vitamin D produces excellent calcification on either a low-phosphorus or a high-phosphorus basal diet. We have also used this salt mixture with apparent success for reproduction studies in mice on both low-phosphorus and high-phosphorus basal diets.

## SUMMARY

The composition of a complete salt mixture (salt mixture 12) satisfactory for use with purified basal diets either low or high in phosphorus is given. This mixture has been compared with salt mixture 351 of Hubbell, Mendel and Wakeman at levels of 2, 3 and 4 gm. per 100 gm. of basal diet. With a basal diet low in phosphorus, the new salt mixture was definitely superior to salt mixture 351 for both calcification and growth at all levels fed. With a basal diet containing liberal amounts of phosphorus, 2 gm. of salt mixture 351 were slightly better than an equal amount of salt mixture 12, but at the 3- and 4-gm. levels there was no detectable difference. When fed at either 3 or 4 gm. and in the presence of liberal amounts of vitamin D the salt mixture described gives satisfactory results with either a phosphorus-high or a phosphorus-low basal diet. In the latter case, however, 3 gm. per 100 gm. of basal diet appears to be a border-line amount and under these conditions the feeding of 4 gm. of the mixture is recommended. At this level the salt mixture described gives very satisfactory results regardless of the amount of phosphorus in the basal diet.

### LITERATURE CITED

BETHKE, R. M., C. H. KICK AND W. WILDER 1932 The effect of the calcium-phosphorus relationship on growth, calcification, and blood composition of the rat. J. Biol. Chem., vol. 98, p. 389.

CLARK, E. P., AND J. B. COLLIP 1925 A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., vol. 63, p. 461.

DANIELS, A. L., AND G. J. EVERSON 1935 The relation of manganese to congenital debility. J. Nutrition, vol. 9, p. 191.

EVANS, R. J., AND P. H. PHILLIPS 1939 A new low fluorine diet and its effect upon the rat. J. Nutrition, vol. 18, p. 353.

GUNTHER, L., AND D. M. GREENBERG 1929 A note on the determination of the inorganic phosphate of the serum on the filtrate from calcium analysis. J. Biol. Chem., vol. 82, p. 551.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, p. 273.

JOHNSON, F. F., AND E. R. FREDERICK 1940 Stabilization of iodine in salt and feedstuffs. Science, vol. 92, p. 315.

JONES, J. H.   1938   The use of fibrin in synthetic diets. J. Nutrition, vol. 15, p. 269.
────────   1939   A study of rachitogenic diets composed of purified food materials. J. Nutrition, vol. 17, p. 601.
PHILLIPS, P. H., AND E. B. HART   1935   The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., vol. 109, p. 657.
RICHARDSON, L. R., AND A. G. HOGAN   1940   Adequacy of a milk diet for the rat. J. Nutrition, vol. 19, p. 13.
SHOHL, A. T.   1936   Rickets in rats. XV. The effect of low calcium-high phosphorus diets at various levels and ratios upon the production of rickets and tetany. J. Nutrition, vol. 11, p. 275.
SURE, B.   1940   The essential nature of choline for lactation and growth of the albino rat. J. Nutrition, vol. 19, p. 71.
────────   1941   Dietary requirements for fertility and lactation. XXIX. The existence of a new dietary factor essential for lactation. J. Nutrition, vol. 22, p. 499.
WAISMAN, H. A., O. MICKELSEN, J. M. McKIBBIN AND C. A. ELVEHJEM   1940   Nicotinic acid potency of food materials and certain chemical compounds. J. Nutrition, vol. 19, p. 483.

# THE SULFUR METABOLISM OF CHILDREN [*]

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FOUR FIGURES

Information relating to sulfur metabolism in growing children has been limited. Schwarz ('10) has presented detailed data for a 5-year-old child. Folin ('05) in his classic paper and, later, Clark ('26) explored the over-all sulfur economy and urinary excretion in adults. An opportunity to examine the sulfur retention, excretion and urinary sulfur partition of the normal child, in relation to nitrogen metabolism, was presented during a detailed, continuous, 45-day metabolic balance study of eight children, 8 to 12 years of age. The results contribute information concerning the manner in which sulfur is metabolized and retained by children.

## EXPERIMENTAL

The subjects studied were normal, healthy children [1] as judged by past nutritional history, hematological observations, growth rate and detailed x-ray studies of skeletal maturity. Seven of the subjects were boys; one girl, subject III, was

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[*] Some of the data in this paper were included in a report given before the Division of Biological Chemistry of the American Chemical Society at the national meeting in Cincinnati, Ohio, April 8–12, 1940.

[1] One child, subject I, deviated from normal only in having an essential idiopathic lipemia (Bernstein, Williams, Hummel, Shepherd and Erickson, '39). Although originally not under study for interpretation with the normal children, it was found that this subject fitted into the general picture of chemical growth presented by the other children, and therefore he has been included in the normal group for the purposes of the present discussion.

included in the group. Throughout the study, the children lived at the Methodist Children's Village, Detroit, under conditions of healthful activity and emotional security, the only conditions under which observations of normal metabolism are possible.

The diet was made up of twenty-two common foods, given daily and in constant quantity for each child throughout the study, at a caloric level suitable for normal growth (Macy, '42). The diet furnished adequate supplies of minerals, proteins, vitamins and all known nutritional essentials for physical health and well-being.

The study was divided into 5-day balance periods. Samples of the mixed dietary were analyzed each period, for nitrogen by the Kjeldahl method and for sulfur after combustion of vacuum-dried samples in the Parr oxygen bomb. All samples of urine were collected quantitatively and held, under toluene, in a refrigerator until the 5-day collection was complete. Carmine was given at the end of each period, to mark the feces of separation. Urinary sulfur partitions were obtained by the methods of Folin ('05–'06) and Denis ('10–'11). Sulfur partition studies were instituted following two 5-day periods which allowed time both for some physiological adjustment to the experimental regimen and for establishing the metabolic routine so that accuracy of the collecting technique was assured. During the subsequent nine 5-day periods, composite samples of urine were used to determine the sulfur partition.

In table 1 the average daily values for each of the individuals are presented, with their standard deviations. The data recorded are the means of averages of duplicate or triplicate analyses of samples collected within the 5-day periods and carry considerably more weight than single, isolated determinations. Errors inherent in the method of determining the sulfur partition, particularly with neutral and ethereal sulfate, tend to be compensated in the mean values. In table 1 the subjects are arranged in order of increasing age, which is also the sequence of increasing body weight.

*Average daily nitrogen and sulfur balances and urinary sulfur partition during 45 consecutive days.*[1]

| | I | II | III | IV | V | VI | VII | VIII |
|---|---|---|---|---|---|---|---|---|
| Age—5th period, months | 103 | 104 | 112 | 114 | 127 | 132 | 143 | 147 |
| Body weight—5th period, kg. | 24.49 | 26.94 | 28.32 | 29.12 | 31.84 | 33.70 | 35.82 | 41.85 |
| Total weight gain—1st–9th period, kg. | 1.28 | 0.30 | 2.89 | —0.96˙ | 1.01 | 0.65 | 1.10 | 0.92 |
| Recumbent length—7th period, cm. | 120.4 | 132.2 | 131.3 | 140.9 | 139.8 | 145.2 | 138.3 | 159.3 |
| **Basal heat production** | | | | | | | | |
| Total (Cal./hr.) | 54.0 | 51.4 | 54.5 | 60.9 | 56.1 | 57.1 | 55.7 | 59.8 |
| Cal./sq.m./hr. | 61.4 | 51.1 | 54.0 | 56.2 | 50.1 | 48.2 | 47.4 | 43.4 |
| **Laxation rate** (defecations per day) | 1.3 | 1.2 | 1.1 | 1.7 | 1.0 | 0.9 | 1.0 | 0.7 |
| | ±0.2 | ±0.2 | ±0.2 | ±0.3 | ±0.1 | ±0.3 | ±0.2 | ±0.2 |
| **AVERAGE PER DAY** | | | | | | | | |
| *Intake* | | | | | | | | |
| Nitrogen, gm. | 13.17 | 13.17 | 13.17 | 13.17 | 13.38 | 13.38 | 13.38 | 13.38 |
| | ±.22 | ±.22 | ±.22 | ±.22 | ±.22 | ±.22 | ±.22 | ±.22 |
| Sulfur, mg. | 977 | 977 | 977 | 977 | 1003 | 1003 | 1003 | 1003 |
| | ±58 | ±58 | ±58 | ±58 | ±58 | ±58 | ±58 | ±58 |
| Ratio nitrogen/sulfur [2] | 13.5 | 13.5 | 13.5 | 13.5 | 13.3 | 13.3 | 13.3 | 13.3 |
| *Fecal outgo* | | | | | | | | |
| Nitrogen, gm. | 1.43 | 1.12 | 1.19 | 1.54 | 1.15 | 1.21 | 1.43 | 1.45 |
| | ±.03 | ±.11 | ±.07 | ±.16 | ±.07 | ±.23 | ±.09 | ±.14 |
| Sulfur, mg. | 135 | 106 | 118 | 145 | 115 | 113 | 133 | 138 |
| | ±9 | ±10 | ±7 | ±8 | ±10 | ±16 | ±11 | ±10 |
| Ratio nitrogen/sulfur [2] | 10.6 | 10.5 | 10.1 | 10.6 | 10.0 | 10.7 | 10.7 | 10.5 |
| *Absorption* [3] | | | | | | | | |
| Nitrogen, gm. | 11.74 | 12.05 | 11.98 | 11.63 | 12.23 | 12.17 | 11.95 | 11.93 |
| Per cent of intake | 89.1 | 91.5 | 91.0 | 88.3 | 91.4 | 91.0 | 89.3 | 89.2 |
| Sulfur, mg. | 842 | 871 | 859 | 832 | 888 | 890 | 870 | 865 |
| Per cent of intake | 86.2 | 89.2 | 87.9 | 85.2 | 88.5 | 88.7 | 86.7 | 86.2 |
| Ratio nitrogen/sulfur [2] | 13.9 | 13.8 | 13.9 | 14.0 | 13.8 | 13.7 | 13.7 | 13.8 |
| *Urinary outgo* | | | | | | | | |
| Nitrogen, gm. | 10.96 | 11.26 | 10.55 | 10.82 | 11.10 | 11.21 | 11.02 | 11.02 |
| | ±.37 | ±.39 | ±.47 | ±.45 | ±.12 | ±.19 | ±.43 | ±.37 |
| Sulfur, mg. | 750 | 784 | 726 | 746 | 773 | 793 | 776 | 754 |
| | ±23 | ±26 | ±25 | ±28 | ±26 | ±18 | ±23 | ±20 |
| Ratio nitrogen/sulfur [2] | 14.6 | 14.4 | 14.5 | 14.5 | 14.4 | 14.1 | 14.2 | 14.6 |
| *Retention* | | | | | | | | |
| Nitrogen, gm. | 0.78 | 0.79 | 1.43 | 0.81 | 1.13 | 0.96 | 0.93 | 0.91 |
| | ±.16 | ±.10 | ±.29 | ±.28 | ±.30 | ±.21 | ±.30 | ±.10 |
| Sulfur, mg. | 92 | 87 | 133 | 86 | 115 | 97 | 94 | 111 |
| | ±65 | ±57 | ±66 | ±63 | ±59 | ±68 | ±75 | ±66 |
| Ratio nitrogen/sulfur [2] | 8.5 | 9.0 | 10.8 | 9.4 | 9.9 | 9.9 | 9.9 | 8.2 |
| *Urinary sulfur partition* | | | | | | | | |
| Inorganic SO₄, mg. | 655 | 676 | 622 | 620 | 644 | 664 | 673 | 604 |
| | ±15 | ±15 | ±23 | ±21 | ±17 | ±16 | ±19 | ±16 |
| Ethereal SO₄, mg. | 43 | 42 | 39 | 42 | 50 | 54 | 43 | 68 |
| | ±10 | ±6 | ±4 | ±7 | ±5 | ±8 | ±4 | ±4 |
| Neutral sulfur, mg. | 52 | 66 | 65 | 84 | 79 | 75 | 60 | 82 |
| | ±16 | ±8 | ±11 | ±16 | ±12 | ±11 | ±6 | ±14 |
| *Per cent of total urinary sulfur* | | | | | | | | |
| Inorganic SO₄ | 87.4 | 86.2 | 85.7 | 83.1 | 83.3 | 83.7 | 86.8 | 80.1 |
| Ethereal SO₄ | 5.7 | 5.4 | 5.4 | 5.6 | 6.5 | 6.8 | 5.5 | 9.0 |
| Neutral sulfur | 6.9 | 8.4 | 8.9 | 11.3 | 10.2 | 9.5 | 7.7 | 10.9 |

[1] Values given are means or means plus or minus standard deviations.
[2] Ratios were calculated from milligrams of nitrogen and sulfur.
[3] Calculated as the intake minus fecal outgo of each element.

*Intake and absorption*

The four younger children received daily a diet containing 0.973 ± 0.043 gm. sulfur and 13.09 ± 0.39 gm. nitrogen, while the older children, who received a similar diet containing additional calories in the form of potato and washed sweet butter, consumed 0.999 ± 0.037 gm. sulfur and 13.27 ± 0.48 gm. nitrogen. Thus, the diets were designed to satisfy the needs of the subjects for all nutriments, but were almost identical in the quantities of nitrogen and sulfur furnished.

With the metabolic balance technique it is not possible to determine the actual amount of sulfur or nitrogen absorbed by the body. This difficulty arises because of the complex nature of the fecal losses. How much fecal nitrogen and sulfur, if any, was absorbed and re-excreted into the intestine is impossible to determine. In metabolic procedure the closest indication of nitrogen and sulfur absorption is obtained by calculation of the intake minus fecal outgo.

The per cent absorption of nitrogen was consistently greater than that of sulfur, resulting in a slightly higher nitrogen-to-sulfur ratio for the absorption than for the intake. Whether or not this was due to either preferential utilization of nitrogen on the part of the body, an excretion of sulfur into the intestine, or the seizure of sulfur compounds by intestinal flora, is not known. There is remarkable constancy among the nitrogen-to-sulfur ratios for the absorption. A fairly strict parallelism of the nitrogen and sulfur absorption is evident in figure 1, in which the per cent of nitrogen intake absorbed is plotted against the corresponding values for sulfur.

*Excretion of nitrogen and sulfur in urine and feces*

In all phases of metabolism there appear to be parallelisms between nitrogen and sulfur. They occur together in the proteins of the foods and are stored together in the body proteins. While there are many compounds of significance in body economy which contain only one or the other of these elements, the great preponderance of both nitrogen and sulfur storage goes into the construction of proteins.

Most of the dietary nitrogen and sulfur lost from the body appears in the urine. The urinary sulfur loss in our subjects averaged 77.0% of the intake and the urinary nitrogen loss was 82.8% of the intake. The feces, on the other hand, contained only 12.7% and 9.9%, respectively, of the sulfur and nitrogen intakes. Thus, the urine contains five to seven times as much sulfur and seven to ten times as much nitrogen as do the
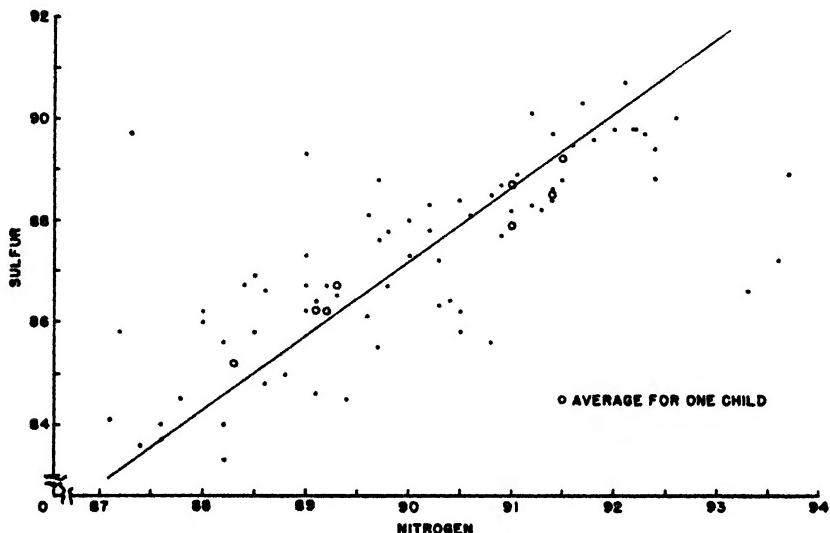


Fig. 1  Relationship between per cent of nitrogen intake absorbed and per cent of sulfur intake absorbed. Dots represent averages for single subjects for 5-day periods; circles are the means of nine 5-day periods for each child.

feces. This is essentially in agreement with observations on adult men (Clark, '26).

The nitrogen-to-sulfur ratios for the urine averaged 14.4; for the feces, 10.5; for the dietary, 13.4. This shift in the nitrogen-to-sulfur ratio during passage of the residue through the alimentary canal is the result of the total of the processes of gastrointestinal secretion, excretion, absorption and bacterial growth which result in the complex fecal residue.

### Urinary sulfur partition

*Inorganic sulfate.* By far the greatest quantity of the sulfur lost in urine is in the form of inorganic sulfates. The children

in this study excreted an average of 84% of their total urinary
sulfur in this form. As many have pointed out, the percentage
lost as inorganic sulfate is variable, dependent primarily on
the level of sulfur intake. On the other hand, ethereal and
neutral sulfur are influenced less by diet and tend to remain
more nearly constant.

*Ethereal sulfur.* Urinary ethereal sulfate has been studied
with interest because of its relation to toxic compounds. The
compounds comprising this group are indole, skatole and
phenol, and perhaps other substances conjugated with sulfuric
acid and excreted as the potassium salt. These residues are
believed to arise from the breakdown of the corresponding
amino acids, tryptophane, phenylalanine and tyrosine. Studies
have indicated that the source of these toxic substances is two-
fold: from some normal, minor, but constant metabolic proc-
esses, and from putrefactive action of the bacteria in the
intestine. The liver has been shown by Pelkan and Whipple
('22) to be the site of this detoxication process.

Indican is the ethereal sulfate of indole. Folin has shown
that, while the level of indole sulfuric acid in the urine is
perhaps a fair index of the extent to which intestinal putrefac-
tion is taking place, there is poor correlation of total ethereal
sulfate sulfur of the urine with urinary indican levels. Dubin
('16) has found that in cases of severe intestinal obstruction
and other disturbances of normal protein digestion there is
an increase in both free and conjugated phenols, and suggests
that total phenol excretion can be used as a measure of in-
testinal putrefaction. In Folin's studies ('05) the ethereal
sulfate level in the urine of adults consuming a mixed dietary
was found to range from about 60 to 100 mg. per day. Schwarz
('10) found in the 5-year-old child an average of 23 mg. per
day. The ethereal sulfate excretion by our 8- to 12-year-old
group averaged 48 mg., which is a level between the adult and
the younger child and indicates a graded output of ethereal
sulfate, increasing with age and perhaps influenced by intensity
of protein metabolism. An examination of the individual values
from our study reveals that the younger children excreted
ethereal sulfate at a slightly lower level than did the older

ones, although they consumed essentially the same quantity of protein.

A marked deviation with age from the slow upward trend of ethereal sulfate is seen in subject VIII, and is suggestive that growth, age or level of protein intake are not the only factors determining urinary ethereal sulfate excretion. In looking for factors to explain this deviation further, we have
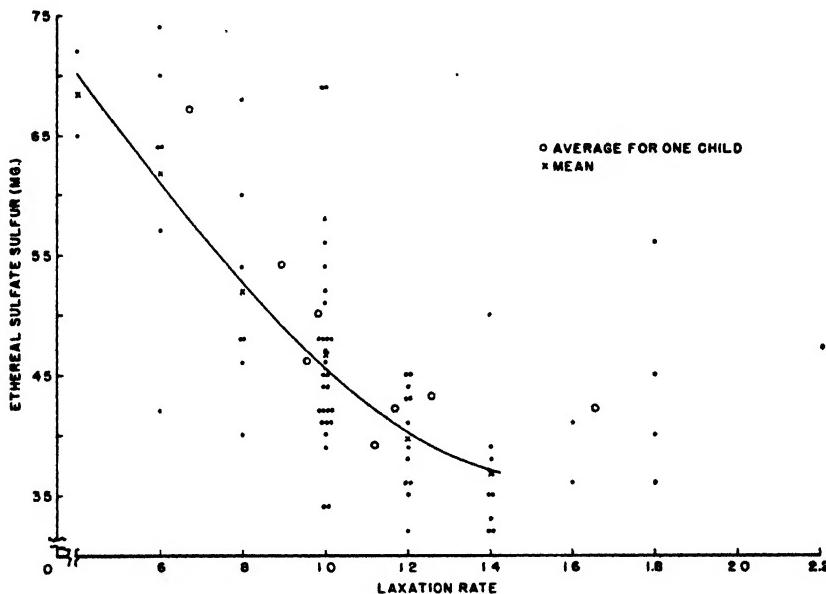


Fig. 2 Relationship between urinary ethereal sulfate sulfur and laxation rate (bowel movements per day). Dots represent averages for single subjects for 5-day periods; circles are the means of nine 5-day periods for each child; crosses are the means for all subjects showing a given laxation rate.

recorded the average laxation rate (bowel movements per day) of each child, and with increasing age there was generally a decreasing laxation rate, although subject IV, in the middle-age group, deviates from this general trend in having a high laxation rate. In figure 2 the urinary ethereal sulfate is plotted against laxation rate and shows the upward trend in ethereal sulfate, with lower laxation rates. It is possible that a low laxation rate with longer retention of feces may be accompanied by a considerable increase in the ethereal sulfate

fraction of the urine, owing to more extensive intestinal putrefaction.

A study of laxation rate, ethereal sulfate excretion and urinary indican should be carried out on enough subjects of sufficiently widely varying laxation rates to provide an adequate test for the relationships which may exist between these physiologic manifestations. That intestinal flora acts more extensively on the fecal residue in subjects of low laxation rate is demonstrated by studies in this laboratory (unpublished data), in which the amounts of food cellulose and hemicellulose disappearing during passage through the gastrointestinal tract were found to be an inverse function of the laxation rate.

*Neutral sulfur.* Neutral sulfur is the fraction of total urinary sulfur which cannot be accounted for as either inorganic or ethereal sulfate sulfur. Inasmuch as the value is obtained by difference and is normally only a small fraction of the total urinary sulfur, the individual determinations tend to be rather unreliable. During the last 5-day period of observation with our subjects, the urinary sulfur partition was studied daily. Wide fluctuations occurred in the neutral sulfur values for each individual and each child varied within a more or less characteristic range. Undoubtedly, some of the variation was due to errors inherent in the method of analysis (Folin, '05); therefore, it is important to obtain a number of observations sufficient for averaging before interpretation is undertaken. Folin suggested that the neutral sulfur fraction reflects constant endogenous metabolic phenomena and demonstrated that it is little influenced by ordinary changes in dietary protein levels. Brody et al. ('34) point out the observation of Amann and Mourot that an increase in urinary neutral sulfur excretion can be produced when the protein intake is increased manyfold above the normal level.

The neutral sulfur fraction is made up of a variety of organic sulfur compounds which normally appear in small amounts in the urine. Among these is cystine, which according to Medes ('36) in the normal subject may contribute as much as 9 mg. of sulfur daily. A portion of the neutral sulfur also may be accounted for as the sulfur of the urinary pig-

ments and thiosulfates, thiocyanates, taurine, mercaptans, and mercapturic acid derivatives. The fraction is of a complex nature and little is known of the forms of sulfur which constitute it.

The average daily level of neutral sulfur output in the age group 8 to 12 years (table 1) was 70 mg., which is higher than the 45 mg. value reported by Schwarz ('10) for the 5-year-old child and is in agreement with Brody's (Brody, Procter and Ashworth, '34) prediction tables for subjects in the weight range studied. The range of results in normal adults, according to Folin ('05), was 52 to 76 mg. daily.
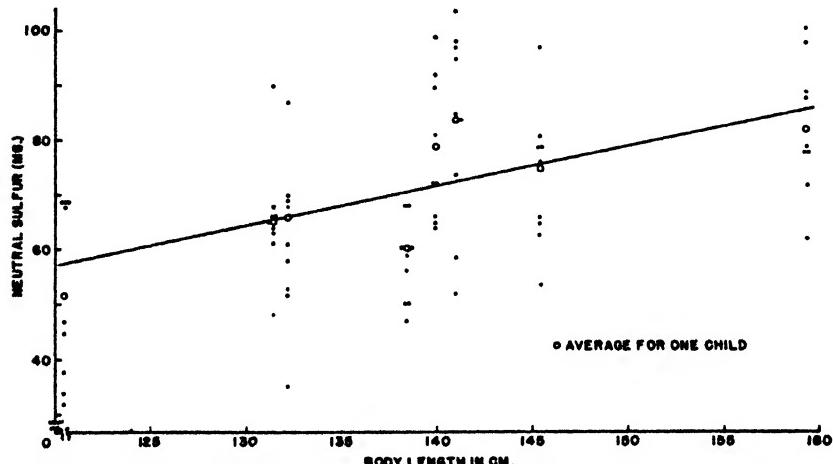


Fig. 3 Relationship between urinary neutral sulfur output and body length. Dots represent averages for single subjects for 5-day periods; circles are the means of nine 5-day periods for each child.

Our observations with the eight children show not more than a remote relationship between neutral sulfur output and age. Brody et al. ('34), studying a variety of animals weighing from 0.02 kg. to 1000 kg., demonstrated a power relationship between body weight and neutral sulfur output. It is not surprising, however, with a limited number of subjects within a restricted weight range, to find variations between neutral sulfur and body weight which may represent only normal deviations within a broad general law of relationship.

Figure 3 shows that there was a tendency in our subjects toward relationship between urinary neutral sulfur and re-

cumbent body length. In contrast, there was a lack of relationship with either body weight or surface area. It is of interest to recall that Daniels (Daniels and Hejinian, '29) has suggested the use of a creatinine-height coefficient, for there is greater constancy of creatinine output per unit height than per unit body weight. While body length and neutral sulfur output may be linked directly, or through interrelation with some common factors, the urinary neutral sulfur is undoubtedly influenced by many other, more complex factors which determine the individual's whole metabolic performance.

Inasmuch as urinary neutral sulfur excretion may reflect endogenous metabolic performance, various measures such as basal heat production, body surface, and creatinine output might be expected to correlate well with level of neutral sulfur. In the present study the relationship is not clearly defined; however, the general upward trend of neutral sulfur excretion with the indices of increased scale of metabolism is undeniable. Basal heat output shows some variations which coincide with those of neutral sulfur. Subject IV had a high basal heat production for his age, equal to that of subject VIII, who was 3 years his senior and 12.5 kg. heavier. Accompanying the equivalent heat production, the two children had the same neutral sulfur output in their urine. On the other hand, subject VII was short and had a somewhat low basal heat output for his size, with a rather low neutral sulfur output. The results for this urinary fraction illustrate the complexity of the various mechanisms which contribute to the end results of sulfur metabolism.

## Sulfur and nitrogen retention

The levels of nitrogen and sulfur retention for the children were not related to age or body size. The greatest retention of these elements occurred in subjects III and V, in the middle of the group, with reference to age and body weight. There seems to be no constant relationship between weight gains and retention values, although the most rapidly growing subject, III, showed the greatest sulfur and nitrogen re-

tentions. The frequent lack of relationship between various indices of growth serves to re-emphasize the fact that no single measurement of a physical attribute or phase of chemical growth over a limited period of time is a reliable criterion for judging the rate at which the complex process of growth and development is progressing.

The greater portions of the nitrogen and sulfur stored by the growing organism are simultaneously incorporated into body protein. For this reason, it is to be expected that their
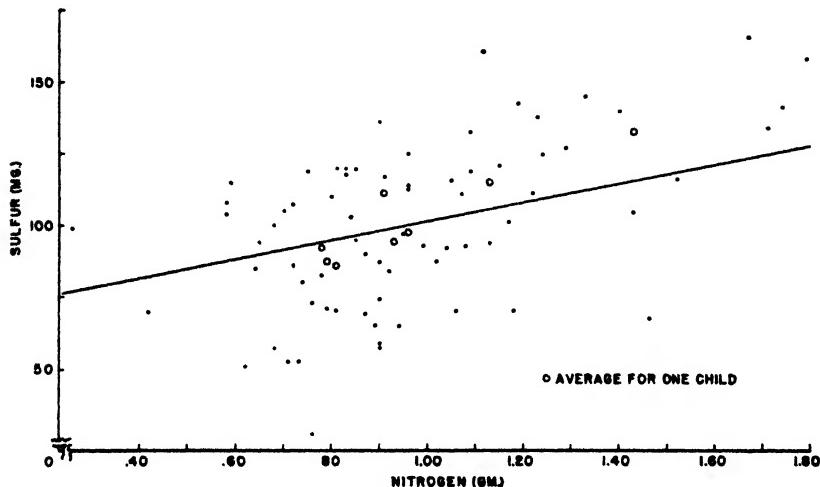


Fig. 4   Relationship between sulfur retention and nitrogen retention. Dots represent averages for single subjects for 5-day periods; circles are the means of nine 5-day periods for each child.

retention would run almost parallel. That this is true to some extent is shown in figure 4, in which retention of nitrogen is plotted against that of sulfur, but the variabilities are more evident from study of the nitrogen-to-sulfur ratios of the retentions (table 1). This ratio varied from 8.2 to 10.8 and averaged 8.8.

Retention is determined in a metabolic balance study as the intake of an element minus the sum of urinary and fecal outgo. Observed retention and true retention are equal only when urine and feces are the only routes by which an element is

lost to the body. In the dog, essentially a non-sweating animal, this would be nearly true. In the human subject, losses of some substances through the skin may be highly significant; this has been shown by Swanson and Iob ('33) with infants. However, in the classical metabolic balance these losses would be included in the retention value. Observations on growing dogs have revealed that the nitrogen-to-sulfur retention ratio is commonly 14.5, and Lewis ('16) has shown during fasting and consequent tissue catabolism a urinary nitrogen-to-sulfur ratio of 13 to 16. In the studies of Deuel et al. (Deuel, Sandiford, Sandiford and Boothby, '28), in one human subject the urinary nitrogen-to-sulfur ratio varied from 5 to 50, over a long period of protein deprivation.

The few analyses available on the nitrogen and sulfur content of the whole body, summarized by Shohl ('29), reveal that newborn infants have a nitrogen-to-sulfur ratio of 8.7, while in adults it is 18.8. Studies in this laboratory, of the nitrogen and sulfur contents of both mixed muscle protein and the protein residues of many soft organs, have shown a nitrogen-to-sulfur ratio of about 15. There are points of concentration of nitrogen and sulfur in the body other than muscle and soft organs which might result in an over-all nitrogen-to-sulfur ratio other than 15 for the whole body. However, Peters and Van Slyke ('31) state that "no matter what may be the ratio of N:S in the dietary protein, the proportions of the two elements retained in the body tend to remain about 14.5:1, the ratio characteristic of animal protein."

Little has been done on the losses of substances through the skin of human subjects. McCance ('36) has shown that sodium chloride losses through the skin during heavy perspiration may cause severe depletion of sodium chloride from the body. Swanson and Iob ('33) revealed that skin losses of mineral elements by the human infant, particularly with reference to potassium, are highly significant even under normal conditions of perspiration and must be measured if true storage of such elements is to be accurately estimated by the metabolic balance technique. More recently, Freyberg and Grant ('37)

determined the skin losses of two adults during periods in which no visible sweating took place. Under these conditions the subjects lost an average of 88 mg. of sulfate sulfur and 200 mg. of soluble nitrogen per 24 hours. The average surface area of the subjects [2] was 1.93 sq.m., which would indicate an average daily loss of 46 mg. of sulfur and 104 mg. of nitrogen per square meter of body surface.

Applying the above results to the group of children in the present study, our subjects may have lost through the skin from 41 to 63 mg. of sulfate sulfur and from 93 to 144 mg. of soluble nitrogen every 24 hours. The observed sulfur retentions varied from 86 to 133 mg. per 24 hours. If the calculated skin losses are correct they represent nearly 50% of the retention value for sulfur. On the other hand, the possible skin loss of nitrogen is of less significance in a retention of 780 to 1130 mg. If these assumptions are correct, therefore, the ratio of nitrogen to sulfur in the true retention of our children may have been considerably higher than the observed retention would indicate. Perhaps future investigations of skin excretion will clarify the interpretation of the results of metabolic studies on these and other elements.

### SUMMARY

During continuous metabolic balance studies of eight normal children, 8 to 12 years old, over 45 days, under controlled conditions of healthful activity and emotional security, sulfur excretion and retention and urinary sulfur partition were studied in relation to nitrogen metabolism. The elimination in the urine represented 77.0 and 82.8% of the sulfur and nitrogen intakes, respectively. Inorganic sulfur composed 84% of the urinary excretion of that element. The average daily excretion of ethereal sulfur was 48 mg., and of neutral sulfur, 70 mg. With increasing age there was generally a decreasing laxation rate, accompanied by increasing urinary ethereal sulfur. Under the conditions of this experiment, only remote relationships were found between neutral sulfur output and

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[2] Personal communication.

age, body weight and surface area, but there was a relation between neutral sulfur and recumbent body length.

The average nitrogen-to-sulfur ratios for intake, feces and urine were 13.4, 10.5, and 14.4, respectively. The nitrogen-to-sulfur ratio of the retentions averaged 8.8; however, if the determined sulfur retentions included losses through the skin comparable to values reported in the literature, the average nitrogen-to-sulfur ratio of the true retentions would be raised to a figure nearer the ratio of 15 which has been indicated for the entire body by analyses of muscle tissue.

LITERATURE CITED

BERNSTEIN, SAMUEL S., HAROLD H. WILLIAMS, FRANCES C. HUMMEL, MARION L. SHEPHERD AND BETTY NIMS ERICKSON 1939 Metabolic observations on a child with essential hyperlipemia. J. Pediatrics, vol. 14, pp. 570–580.

BRODY, S., R. C. PROCTER AND U. S. ASHWORTH 1934 Growth and development with special reference to domestic animals. XXXIV. Basal metabolism, endogenous nitrogen, creatinine and neutral sulphur excretions as functions of body weight. University of Missouri Agricultural Experiment Station Research Bulletin 220.

CLARK, G. W. 1926 Studies in the mineral metabolism of adult man. University of California Publications in Physiology, vol. 5, pp. 195–287.

DANIELS, AMY L., AND LUCEA M. HEJINIAN 1929 Growth in infants from the standpoint of physical measurements and nitrogen metabolism. I. Creatinine. Am. J. Dis. Child., vol. 37, pp. 1128–1134.

DENIS, W. 1910–1911 The determination of total sulphur in urine. J. Biol. Chem., vol. 8, pp. 401–403.

DEUEL, H. J., IRENE SANDIFORD, KATHLEEN SANDIFORD AND W. M. BOOTHBY 1928 A study of the nitrogen minimum. The effect of sixty-three days of a protein-free diet on the nitrogen partition products in the urine and on the heat production. J. Biol. Chem., vol. 76, pp. 391–406.

DUBIN, H. 1916 Physiology of the phenols. J. Biol. Chem., vol. 26, pp. 69–91.

FOLIN, O. 1905 Laws governing the chemical composition of urine. Am. J. Physiol., vol. 13, pp. 66–115.
———— 1905–1906 On sulphate and sulphur determinations. J. Biol. Chem., vol. 1, pp. 131–159.

FREYBERG, R. H., AND R. L. GRANT 1937 Loss of minerals through the skin of normal humans when sweating is avoided. J. Clin. Invest., vol. 16, pp. 729–731.

LEWIS, H. B. 1916 The metabolism of sulfur. I. The relative eliminations of sulfur and nitrogen in the dog in inanition and subsequent feeding. J. Biol. Chem., vol. 26, pp. 61–68.

McCance, R. A. 1936 Experimental sodium chloride deficiency in man. Proc. Roy. Soc. London, Series B, vol. 119, pp. 245–268.

Macy, Icie G. 1942 Nutrition and Chemical Growth in Childhood. Vol. I. Evaluation. Charles C. Thomas, Springfield, Illinois.

Medes, Grace 1936 Metabolism of sulphur. II. The quantitative determination of cystine in normal urine. Biochem. J., vol. 30, pp. 1293–1297.

Pelkan, K. F., and G. H. Whipple 1922 Studies of liver function. III. Phenol conjugation as influenced by liver injury and insufficiency. J. Biol. Chem., vol. 50, pp. 513–526.

Peters, J. P., and D. D. Van Slyke 1931 Quantitative Clinical Chemistry. Vol. I. Interpretations. The Williams and Wilkins Co., Baltimore.

Schwarz, H. 1910 Der Stickstoff- und Schwefelstoffwechsel in Fällen von rachitischem Zwergwuchs und ein Beitrag zum normalen Stoffwechsel eines fünf Jahre alten Knaben. Jahrb. Kinderheilk., vol. 72, pp. 549–574.

Shohl, A. T. 1939 Mineral Metabolism. Reinhold Publishing Co., New York City.

Swanson, W. W., and L. Vivian Iob 1933 Loss of minerals through the skin of infants. Am. J. Dis. Child., vol. 45, pp. 1036–1039.

# THE EFFECT OF PANTOTHENIC ACID DEFICIENCY ON THE BLOOD LIPOIDS OF THE DOG

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A marked depression occurs in the concentration of the blood lipoids as the result of pantothenic acid deficiency in the dog. The blood levels are rapidly restored by the administration of the vitamin.

## EXPERIMENTAL

Cholesterol and cholesterol esters were determined by Bloor's method (Bloor, '16; Bloor and Knudson, '16). Lipoid phosphorus was determined according to Youngburg and Youngburg ('30) and total lipoids were determined by evaporating to dryness an aliquot of the Bloor filtrate. The residues were extracted with petroleum ether, and the extracts were evaporated and dried to constant weight. These analyses were performed with whole blood in the experiments with puppies, and with plasma in the experiments with the adult dogs. Blood was drawn from fasting animals. Analytical data are given in milligrams per cent.

Twenty-seven dogs (four groups consisting of twelve mongrel puppies, six litter-mate setter puppies, four young adult litter-mate setters and five adult mongrels) were given the same basal ration having a percentage composition of vitamin free casein, 30; dextrose, 41; hydrogenated cottonseed oil,[1] 21; corn oil, 0.15; U.S.P. XI salt mixture, 3.0; bone ash, 2.85 and cod liver oil, 2.0. Control animals were given a

[1] Crisco.

daily supplement consisting of 1 mg. each of thiamine, ribo-
flavin, and pyridoxine, 20 mg. of nicotinic acid, 40 mg. of cal-
cium pantothenate, 200 mg. of choline chloride, and 200 mg.
of inositol. Fifty milligrams of $\alpha$-tocopherol was administered
orally every 2 weeks. Additional control animals were given
this supplement plus 5 gm. of dried whole beef liver. To
produce the deficiency in the test animals, both liver and
pantothenic acid were omitted from the supplement. The sup-
plement was included in the diet daily, but when the food
intake fell off, the supplement was given by stomach tube.

In general, the dogs on the deficient diet were allowed to
eat ad libitum, and the food consumption of the control animals
was restricted to the amounts consumed by the deficient ani-
mals. The relationships between the test animals, their con-
trols and the food intake is given briefly in the appropriate
tables.[2]

*Group 1.* Six litter-mate setter weanlings (8 weeks old)
were placed on their respective diets as shown in table 1.
Sixty-seven days after the dogs were placed on the experi-
mental rations, when the rate of growth and the food intake
of the deficient animals had fallen markedly, blood samples
were drawn and analysed. The deficient animals were then
given 4.0 mg. of calcium pantothenate per kilo per day and
blood samples were taken and analysed at the time intervals
shown in table 1.

*Group 2.* Nine mongrel weanlings (age 45–57 days) were
used as shown in table 2. Forty-two days later, when the
growth rate had decreased and the food intake of the deficient
dogs had declined, blood samples were taken and analysed.
Two of the deficient animals were sacrificed for other studies.
The remaining deficient animal was given 4 mg. of calcium
pantothenate per kilogram body weight per day in the supple-
ment, and analyses were performed as indicated in table 2.

*Group 3.* Four male, litter-mate setters ($6\frac{1}{2}$ months old)
were studied and the changes in the blood lipoids were fol-

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[2] Drs. R. H. Silber and A. O. Seeler will discuss this phase of the problem more
fully in forthcoming communications.

lowed as the animals continued on their respective diets. Dogs
63 and 73 were given the supplement without pantothenic acid,
and were allowed to eat the basal ration ad libitum. The food
intake of dog 65 was restricted to that of dog 63, and dog 71
was restricted to what dog 73 ate voluntarily. These control

TABLE 1 [1]

*Blood lipids of litter-mate puppies in pantothenic acid deficiency.*

| DATE AND DOG NO. | BASAL DIET | SUPPLEMENT CONTAINED | CHOLESTEROL | | RATIO OF FREE CHOLESTEROL TO ESTERS | LIPOID P | TOTAL LIPOIDS |
|---|---|---|---|---|---|---|---|
| | | | TOTAL | ESTERS | | | |
| 3/9/42 | | | | | | | |
| 216 ♀ | Restricted to dog 213 | P.A.[2] | 238 | 117 | 1.0 | 11 | 892 |
| 218 ♂ | Restricted to dog 213 | P.A. and liver | 216 | 109 | 1.0 | 12 | 1042 |
| 214 ♀ | Restricted to dog 215 | P.A. and liver | 266 | 128 | 0.93 | 13 | 892 |
| 217 ♂ | Restricted to dog 215 | P.A. | 221 | 110 | 0.99 | 12 | 908 |
| 213 ♂ | Ad lib. | No P.A. | 124 | 42 | 0.51 | 9 | 483 |
| 215 ♀ | Ad. lib. | No P.A. | 157 | 57 | 0.57 | 10 | 717 |
| 3/14/42 | | | | | | | |
| 213 | Ad lib. | 2 doses of P.A. | 252 | 92 | 1.5 | 16 | 1092 |
| 215 | Ad lib. | 2 doses of P.A. | 198 | 74 | 0.60 | 13 | 833 |
| 3/21/42 | | | | | | | |
| 213 | | Daily doses of P.A. | 226 | 110 | 0.95 | 16 | 967 |
| 215 | | Daily doses of P.A. | 178 | 87 | 0.96 | 14 | 850 |

[1] Analytical data are given in ''mg. per cent.''

[2] P.A. = Calcium Pantothenate.

animals received the supplement plus 4 mg. of pantothenic acid
per day. Blood samples were taken and analysed at the time
intervals shown in table 3.

*Group 4.* This group of animals, used in conjunction with
other experiments, was placed on the basal ration ad libitum
July, 1940. Control animals 76 and 80 were given a daily
supplement consisting of 0.5 to 1.0 mg. each of thiamine, ribo-

flavin, and pyridoxine, and 5 to 10 mg. each of nicotinic acid and pantothenic acid. Neither choline nor inositol was included. Dog 112 was given the same supplement minus panto-

TABLE 2

*Blood lipids in mongrel puppies with pantothenic acid deficiency.*
*Analytical data are in milligrams per cent.*

| DATE AND DOG NO. | BASAL DIET | SUPPLEMENT CONTAINED | CHOLESTEROL | | RATIO | LIPOID P | TOTAL LIPOIDS |
|---|---|---|---|---|---|---|---|
| | | | TOTAL | ESTERS | | | |
| 2/11/42 | | | | | | | |
| 207 ♀ | Restricted to dog 206 | P.A. | 357 | 166 | 0.87 | 18 | 1250 |
| 211 ♀ | Restricted to dog 206 | P.A. and liver | 295 | 132 | 0.81 | 17 | 1133 |
| 201 ♂ | Ad lib. | P.A. | 256 | 126 | 0.97 | 14 | 1107 |
| 202 ♀ | Ad lib. | P.A. | 252 | 120 | 0.91 | 15 | 1160 |
| 203 ♂ | Ad lib. | P.A. | 269 | 132 | 1.0 | 13 | 1147 |
| 204 ♂ | Restricted to dog 201 | P.A. and liver | 306 | 146 | 0.91 | 14 | 1193 |
| 205 ♀ | Restricted to dog 203 | P.A. and liver | 298 | 155 | 1.1 | 14 | 1127 |
| 208 ♂ | Restricted to dog 210 | P.A. | 339 | 158 | 0.81 | 17 | 1225 |
| 209 ♂ | Restricted to dog 210 | P.A. and liver | 339 | 175 | 1.1 | 17 | 1083 |
| 210 ♀ | Ad lib. | No P.A. | 214 | 75 | 0.54 | 14 | 834 |
| 212 ♂ | Ad lib. | No P.A. | 219 | 90 | 0.70 | 14 | 927 |
| 3/11/42 | | | | | | | |
| 206 ♂ | Ad lib. | No P.A. | 224 | 96 | 0.75 | 15 | 1117 |
| 3/13/42 | | | | | | | |
| 206 ♂ | Ad lib. | 2 doses of P.A. | 297 | 133 | 0.81 | 18 | 1150 |
| 3/21/42 | | | | | | | |
| 206 ♂ | Ad lib. | Daily doses of P.A. | 238 | 121 | 1.0 | 17 | 942 |
| 3/21/42 | | | | | | | |
| 211 ♀ | Restricted to dog 206 | P.A. | 258 | 124 | 0.93 | 17 | 1000 |

thenic acid. Dogs 103 and 113 were given the same supplement minus pantothenic acid and riboflavin. In February of the following year these dogs were given riboflavin and were thus continued with a lack of only pantothenic acid. Through the

TABLE 3 [1]

Blood lipids in paired-fed growing dogs (6½ months old) with pantothenic acid deficiency. Analytical data are in milligrams per cent.

| DATE | CHOLESTEROL | | RATIO | LIPOID P | TOTAL LIPOIDS | CHOLESTEROL | | RATIO | LIPOID P | TOTAL LIPOIDS |
|---|---|---|---|---|---|---|---|---|---|---|
| | TOTAL | ESTERS | | | | TOTAL | ESTERS | | | |
| | *Dog 63* | | | | | *Dog 65* | | | | |
| 12/17/41 | 271 | 173 | 1.8 | .. | 1072 | 311 | 185 | 1.5 | 18 | 1000 |
| 1/ 7/42 | 273 | 185 | 2.1 | 18 | 930 | 230 | 156 | 2.1 | 19 | 1173 |
| 1/15/42 | 260 | 153 | 1.4 | 15 | ... | 240 | 155 | 1.8 | 14 | ... |
| 1/28/42 | 282 | 203 | 2.6 | 17 | ... | 270 | 163 | 1.5 | 17 | ... |
| 2/19/42 | 196 | 88 | 0.8 | 14 | 550 | 239 | 145 | 1.5 | 15 | 658 |
| 3/ 7/42 | 181 | 115 | 1.7 | 15 | 893 | 251 | 116 | 0.9 | 19 | 1125 |
| 3/21/42 | 155 | 60 | 0.6 | 11 | 517 | 198 | 157 | 3.8 | 13 | 766 |
| 3/30/42 | 160 | 57 | 0.6 | 9 | 634 | 254 | 131 | 1.1 | 12 | 875 |
| | *Dog 73* | | | | | *Dog 71* | | | | |
| 12/17/41 | 304 | 195 | 1.8 | .. | 999 | 259 | 176 | 2.1 | 18 | 1100 |
| 1/ 7/42 | 273 | 185 | 2.1 | 18 | 980 | 271 | 173 | 1.8 | 17 | 1167 |
| 1/15/42 | 288 | 193 | 2.0 | 19 | ... | 238 | 178 | 3.0 | 16 | ... |
| 1/28/42 | 256 | 162 | 1.7 | 17 | ... | 281 | 217 | 3.4 | 17 | ... |
| 2/19/42 | 290 | 193 | 2.0 | 17 | 790 | 314 | 225 | 2.5 | 20 | 900 |
| 3/ 7/42 | 271 | 185 | 2.2 | 20 | 1100 | 271 | 183 | 2.1 | 19 | 1300 |
| 3/18/42 [2] | 190 | 129 | 2.1 | 15 | 717 | 295 | 181 | 1.6 | 18 | 860 |

[1] Animals placed on test 12/19/41.
[2] Dogs sacrificed in conjunction with other work.

courtesy of Drs. A. O. Seeler and R. H. Silber we studied these dogs from December, 1941 to the present time. The same depressed blood lipoid picture was observed. Upon administration of pantothenic acid (4 mg. per kilogram per day) to dogs 103 and 113, the blood lipoid level increased as shown in table 4.

TABLE 4

*Blood lipids of dogs in pantothenic acid deficiency and receiving various vitamin supplements (see text). Analytical data are in "milligrams per cent."*

| DATE | DOG NO. | CALCIUM PANTO-THENATE IN SUPPLE-MENT | CHOLESTEROL | | RATIO | LIPOID P | TOTAL LIPOIDS |
|------|---------|------|-------|--------|-------|----------|-------|
| | | | TOTAL | ESTERS | | | |
| 12/3/41 | 76 | + | 193 | 109 | 1.3 | 9 | 777 |
| | 80 | + | 187 | 87 | 0.9 | 8 | 600 |
| | 103 | — | 88 | 44 | 1.0 | 4 | 300 |
| | 112 | — | 76 | 37 | 1.0 | 5 | 300 |
| | 113 | — | 120 | 64 | 1.1 | 7 | 503 |
| 12/23/41 [1] | 76 | + | 203 | 93 | 0.9 | 11 | 776 |
| | 80 | + | 109 | 51 | 0.9 | 6 | 470 |
| | 103 | + | 180 | 72 | 0.7 | 10 | 770 |
| | 112 | — | 68 | 34 | 1.0 | 5 | 490 |
| | 113 | + | 188 | 103 | 1.2 | 12 | 867 |
| 2/16/42 | 76 | + | 157 | 83 | 1.1 | 15 | 816 |
| | 80 | + | 161 | 99 | 1.6 | 13 | 750 |
| | 103 | + | 188 | 133 | 2.4 | 17 | 955 |
| | 112 | — | 90 | 47 | 1.1 | 8 | 458 |
| | 113 | + | 168 | 104 | 1.6 | 14 | 800 |

[1] The administration of calcium pantothenate to dogs numbered 103 and 113 was begun 12/14/41.

## DISCUSSION

These results indicate that a deficiency of pantothenic acid in the dog produces a lowering of the blood cholesterol, cholesterol esters, lipoid phosphorus, and total lipoids. The blood levels are rapidly increased by as little as 2 daily doses of the vitamin. Recently Schaeffer, McKibbin and Elvehjem ('42) reported that in their pantothenic acid deficient dogs the blood sugar was usually lowered; the blood chlorides were slightly depressed; non-protein nitrogen was increased, and

serum calcium and inorganic phosphorus remained normal in severely deficient dogs. At post-mortem examination the deficient animals showed fatty livers. In our animals the blood urea appeared normal and the blood sugar was lowered only in dog no. 112. Shortly before collapse, the urea nitrogen rose and the blood sugar fell. In our group of young adult setters, the inorganic phosphorus, chlorides, sodium and potassium were not significantly altered. The striking changes occurred in all of our animals in the blood lipoid picture.

Henderson, McIntire, Waisman and Elvehjem ('42) reported that their excretion studies in the rat indicate a decrease in pantothenic acid requirement as the growth rate decreases. More recently, Unna and Richards ('42) reported a striking decrease with progressing age in the requirement of the rat. In the present study it was not possible to follow the blood changes serially in all the animals, but depressed blood lipoids were noted in pups about 40 to 60 days after the animals were placed on pantothenic acid deficient diets, and collapse rapidly followed. Similar lipoid changes as well as loss of appetite and growth rate developed somewhat more slowly in the young adult setters. Depressed blood lipoids were also observed in the adult mongrels, but it should be noted that the onset of the pantothenic acid deficiency symptoms was distinctly delayed in these animals. In agreement with previous findings in the rat, it would appear that a pantothenic acid deficiency is not as critical in adult dogs as it is in pups.[3]

The studies of Epstein ('31, '32) suggest that the depressed cholesterol-cholesterol ester ratios reported here are indicative of liver damage. Through the courtesy of Dr. William Antopol of the Newark Beth Israel Hospital, who is investigating the pathology of pantothenic acid deficiency, we have the following preliminary report:

Dog no. 210 (female, deficient): almost the entire liver is composed of large vacuolated and granular liver cord cells.[4]

[3] See footnote 2, page 274.

[4] All sections except those designated as stained with sudan III were cut from formalin fixed tissue embedded in paraffin and stained with eosin and hematoxylin.

The sinuses are very narrow and in many places abut against one another. Only a rare area, usually nearer the central vein, is not involved in this process. Some of the liver cells contain 2 nuclei and in places there is a moderate variation in size and pyknocity. The Kupffer cells are difficult to distinguish. The findings are strikingly revealed in frozen sections stained with sudan III. Except for small areas, near the center of the lobules the cells are found to be filled universally with sudanophilic substances.

Dog no. 212 (male, deficient): the vacuoles are finer than those in dog no. 210 and the cytoplasm, in places, is more granular. The extent of the involvement however, is almost as great as that in dog no. 210. The variation in nuclear size and staining is not as evident as in no. 210. Sudan stain shows essentially the same finding as in dog no. 210.

Dog. no. 208 (male, given pantothenic acid): shows areas with numerous larger vacuolated and pale granular cells. These for the most part are localized toward the periphery of the lobules. The remainder of the liver is for the most part composed of smaller cells, with a more eosinophilic cytoplasm which is either non-vacuolated, or contains a moderate number of vacuoles. The sinuses are wider than those observed in dogs nos. 210 and 212 and the Kupffer cells are easily distinguishable. Sudan III stain shows the vacuoles to be filled with globules which stain positively.

Dog no. 209 (male, receiving pantothenic acid and liver): shows only a rare small group of liver cord cells, which contain vacuoles. As a rule these are nearer the periphery of the lobule. The liver cord cells are small and the cytoplasm is eosinophilic, but in some of the cells more intensively staining globules and granules are also present. The sinuses are easily distinguishable as are the Kupffer cells. Many of the latter have a fainter staining cytoplasm than the liver cells proper. The differences between these livers are brought out strikingly by the sudan III stain on frozen sections which shows only rare sudanophilic globules.

Although the most extensive liver changes were found in the pantothenic acid deficient animals, dog no. 208 which received pantothenic acid in addition to the stock supplement still showed considerable fat in the liver, whereas dog no. 209, which received both pantothenic acid and whole dried liver, showed minimal changes. This difference in the control animals suggests the presence of unknown factors in the whole dried beef liver. However, the diminished food consumption makes it necessary to consider the significance of a low protein intake.

## SUMMARY

In experiments involving twenty-seven dogs, the animals were divided into three age groups consisting of weanlings, young adults and adults. Pantothenic acid deficiency was more critical in weanlings than in adult dogs. Ten of the dogs were maintained on pantothenic acid deficient diets, and all ten of the animals showed a lowered blood cholesterol, cholesterol ester, lipoid phosphorus and total lipoids. Five of the deficient animals were given doses of the vitamin, and the blood lipoid levels were rapidly increased.

Dogs receiving pantothenic acid and whole dried liver in addition to the stock supplement showed minimal liver damage, whereas dogs receiving only pantothenic acid showed considerable fat in their livers. Pantothenic acid deficient dogs developed extremely fatty livers.

## LITERATURE CITED

BLOOR, W. R. 1916 The determination of cholesterol in the blood. J. Biol. Chem., vol. 24, p. 227.

BLOOR, W. R., AND ARTHUR KNUDSON 1916 The separate determination of cholesterol and cholesterol esters in small amounts of blood. J. Biol. Chem., vol. 27, p. 107.

EPSTEIN, E. Z. 1931 The cholesterol partition of the blood plasma in parenchymatous diseases of the liver. Arch. Int. Med., vol. 47, p. 82.

———— 1932 Cholesterol of the blood plasma in hepatic and biliary diseases. Arch. Int. Med., vol. 50, p. 203.

HENDERSON, L. M., J. M. McINTIRE, H. A. WAISMAN AND C. A. ELVEHJEM 1942 Pantothenic acid in the nutrition of the rat. J. Nutrition, vol. 23, p. 47.

SCHAEFFER, A. E., J. M. McKIBBIN AND C. A. ELVEHJEM  1942  Pantothenic
        acid deficiency study in dogs. J. Biol. Chem., vol. 143, p. 321.
YOUNGBURG, G. E., AND M. V. YOUNGBURG  1930  Phosphorus metabolism: system
        of blood phosphorus analysis. J. Lab. Clin. Med., vol. 16, p. 158.
UNNA, K., AND G. V. RICHARDS  1942  Relationship between pantothenic acid
        requirement and age in the rat. J. Nutrition. (In press.)

# THE INTERRELATIONSHIP OF CALCIUM, PHOSPHORUS AND NITROGEN IN THE METABOLISM OF PRE-SCHOOL CHILDREN [1]

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Calcium, phosphorus and nitrogen storage represent only a part of the complicated processes which occur during the growth of children. In all probability, there is an interrelationship between the quantity of any one of these minerals deposited and the amount of the others utilized. Mineral deposition in bone could well be regulated by the availability of either calcium or phosphorus. Phosphorus also forms a part of the molecule of certain proteins and if the quantity available for use in the body were limited, this might reduce nitrogen storage and thus curtail muscle growth. Although growth studies indicate that bone takes precedence over muscle tissue growth, it is possible that under some circumstances a large utilization of phosphorus in soft tissue might reduce the amount available for bone production.

In previous publications, Hawks et al. ('37, '38 and '40) reported studies showing the effect of increasing the protein content of the diet on the protein and calorie utilization of pre-school children. At the same time they also determined calcium and phosphorus balances. This paper discusses the

interrelationships between the retentions of calcium, phosphorus and nitrogen.

PROCEDURE

The method of procedure has been discussed in detail (Hawks et al., '37) and therefore only a brief account will be presented here. Two groups of pre-school children received, first, a diet containing 3 and, then, one containing 4 gm. of protein per kilogram of body weight. In the first experiment, the 4-gm. protein diet contained additional milk, part of it skimmed to adjust the calorie value, meat and whole egg, while in the second experiment, the diet had added egg white and gelatin and a reduced quantity of butter. Experimental periods continued for from 15 to 24 days. In both experiments the conditions were carefully controlled. In the first, there was a slight, but probably insignificant, increase in intake of calories, calcium and phosphorus, but in the second, these substances all remained the same on the two diets. The children received adequate quantities of vitamin D daily.[2]

Calcium was determined according to the volumetric procedure of Kramer and Howland ('26). Phosphorus was determined by the uranium titration method of Pincus as outlined by Peters and Van Slyke ('32). Nitrogen was estimated by the official Kjeldahl-Gunning method (Association of Official Agricultural Chemists, '25).

RESULTS

On account of the different method of increasing the protein content of the diet in the two experiments, the results will be discussed separately. They cannot be compared directly with the results of other investigators since the purpose and technic employed were not entirely the same. In order to make direct comparisons, part of the nitrogen values which were previously published (Hawks, Bray and Dye, '38) have

[2] Subjects B and D received 800 and 840 units, respectively, per day, and children V, C and J each had 1700 units per day. These amounts comply with the allowance which the National Research Council's Committee on Foods and Nutrition recently recommended.

been included in the tables with the figures for calcium and phosphorus.

## Experiment 1

*Diet.* Although calcium and phosphorus intakes were higher on the 4- than on the 3-gm. protein diet (table 1), all of them

TABLE 1

*Calcium, phosphorus and nitrogen metabolism data expressed per kilogram of body weight.*

Experiment I.

| MINERAL | | CHILD | 3-GM. PROTEIN DIET | | | 4-GM. PROTEIN DIET | | |
|---|---|---|---|---|---|---|---|---|
| | | | Range | Mean | Proportion of intake [1] | Range | Mean | Proportion of intake [1] |
| | | | mg. | mg. | % | mg. | mg. | % |
| Calcium | Intake | B | 58–61 | 60 | .... | 69–71 | 70 | .... |
| | | D | 57–60 | 59 | .... | 68–70 | 69 | .... |
| | Feces | B | 50–59 | 53 | 88.3 | 60–61 | 61 | 87.4 |
| | | D | 46–56 | 49 | 82.8 | 53–57 | 56 | 81.1 |
| | Urine | B | 2–4 | 2 | 4.2 | 2–3 | 3 | 3.8 |
| | | D | 5–6 | 5 | 9.3 | 6–7 | 7 | 9.8 |
| | Retention | B | (−2)–8 | 5 | 7.5 | 5–8 | 6 | 8.8 |
| | | D | (−2)–8 | 5 | 7.9 | 5–8 | 6 | 9.1 |
| Phosphorus | Intake | B | 64–68 | 66 | .... | 81–86 | 84 | .... |
| | | D | 63–67 | 66 | .... | 81–85 | 83 | .... |
| | Feces | B | 21–24 | 22 | 34.0 | 23–30 | 27 | 32.6 |
| | | D | 15–20 | 18 | 27.7 | 21–27 | 23 | 27.7 |
| | Urine | B | 39–43 | 41 | 62.1 | 49–54 | 51 | 60.6 |
| | | D | 41–46 | 45 | 67.9 | 52–56 | 54 | 64.7 |
| | Retention | B | 1–6 | 3 | 3.9 | 1–7 | 6 | 6.8 |
| | | D | 1–5 | 3 | 4.4 | 6–7 | 6 | 7.6 |
| Nitrogen | Intake | B | 500–533 | 513 | .... | 668–707 | 693 | .... |
| | | D | 492–526 | 508 | .... | 662–699 | 687 | .... |
| | Feces | B | 63–103 | 72 | 14.0 | 59–105 | 88 | 12.8 |
| | | D | 46–64 | 53 | 10.4 | 57–67 | 63 | 9.1 |
| | Urine | B | 379–418 | 403 | 78.4 | 503–587 | 543 | 78.4 |
| | | D | 412–444 | 427 | 84.2 | 542–589 | 572 | 83.2 |
| | Retention | B | 25–54 | 38 | 7.6 | 36–103 | 62 | 8.8 |
| | | D | 18–39 | 28 | 5.4 | 35–90 | 52 | 7.7 |

[1] Percentage calculations were based on total daily figures in order to eliminate errors caused by rounding out the kilogram figures.

fulfilled the generally accepted allowances which Sherman ('41) gives. For calcium the increase amounted to 10 mg. and for phosphorus to 17 and 18 mg. per kilogram of body weight for the two children. Fluctuations in diet values from period to period were extremely small, the coefficients of variation being 2.2 and 2.1% for calcium and phosphorus, respectively, on the 3-gm. protein diet, and 1.3 and 2.3% for the same minerals on the 4-gm. protein diet. The increase in nitrogen intake values on the 4-gm. protein diet was 179 and 180 mg. per kilogram. The figures varied slightly from period to period, the coefficients of variation being 2.3 and 2.0% on the 3- and 4-gm. protein diets, respectively.

*Fecal excretion and absorption.* The data in this experiment show that with increased dietary calcium, phosphorus and nitrogen the apparent absorption of all three elements was greater. The increase in dietary protein may have produced these higher absorption values since the percentages of the intake values absorbed were higher on the 4- than on the 3-gm. protein diet, with the exception of phosphorus for child D which remained unchanged. Fecal calcium, phosphorus and nitrogen values per kilogram increased with the higher protein diet, but these increases were not as great as those in the diet and thus produced a lower proportional fecal output.

*Urinary excretion and retention.* The change in the diet caused increases in the retentions of calcium, phosphorus and nitrogen since these values were higher for both children and represented a larger proportion of the intake figures. The increase in retention of calcium was no greater than the increase in absorption, since both apparent absorption and retention values increased on an average 1.3% of the intake. When more calcium was absorbed the body apparently utilized the extra amount available. Although the milligrams of urinary phosphorus increased, they represented a smaller proportion of the phosphorus intake. Thus the high protein diet was associated with an increased phosphorus retention in contrast to the unchanged calcium retentions. The urinary

nitrogen increased on the 4-gm. protein diet but the proportion of the intake eliminated in this manner was approximately the same on the two diets. Therefore the nitrogen retention increased in the same proportion as did the absorption values.

## Experiment 2

*Diet.* In this experiment adjustments made in changing from the 3- to the 4-gm. protein level were such as to eliminate variation in other dietary constituents, and any changes which occurred in calcium and phosphorus metabolism were probably due to the increase in the nitrogen content of the diet. Table 2 shows that, with the exception of the calcium values for subject V, calcium and phosphorus intakes increased only 1 mg. per kilogram with the change to the higher protein diet. The intake values for calcium and phosphorus were similar to, and the figures for nitrogen were slightly lower than, the corresponding figures for the 3-gm. protein diet of the earlier study. Fluctuations from period to period were similar, the coefficients of variation on the two diets being 4.0 and 3.4% for calcium, 3.6 and 3.4% for phosphorus and 3.8 and 3.2% for nitrogen.

*Fecal excretion and absorption.* In order to make the results comparable with those published in the preceding papers on nitrogen (Hawks, Bray and Dye, '38) and calorie metabolism (Hawks, Voorhees, Bray and Dye, '40), the first 9 days on the 4-gm. protein diet were considered as a preliminary period, and only the values for periods 4 to 8 will be included in the following discussion. The mean values for calcium and phosphorus for all periods and for periods 4 to 8 however, were practically the same.

The data suggest that calcium intake rather than changes in protein content affected calcium absorption. The fecal output for two children increased by the same amount as did the diet values, being 3 mg. per kilogram for subject V and 1 mg. per kilogram for child J. Fecal values for subject C did not change. Absorption figures, therefore, remained almost the same on the 3- as on the 4-gm. protein diet and represented practically the same percentage of the intake values.

TABLE 2

*Calcium, phosphorus and nitrogen metabolism data expressed per kilogram of body weight.*

### Experiment II.

| MINERAL | | CHILD | 3-GM. PROTEIN DIET | | | 4-GM. PROTEIN DIET | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Range | Mean | Proportion of intake [1] | Range | Mean total | Periods 4–8 | Proportion of intake [1] |
| | | | *mg.* | *mg.* | % | *mg.* | *mg.* | *mg.* | % |
| Calcium | Intake | V | 55–58 | 56 | .... | 57–61 | 59 | 59 | .... |
| | | C | 57–61 | 59 | .... | 58–63 | 60 | 60 | .... |
| | | J | 53–57 | 55 | .... | 55–59 | 57 | 56 | .... |
| | Feces | V | 42–49 | 46 | 81.5 | 46–52 | 49 | 49 | 83.2 |
| | | C | 45–49 | 47 | 80.0 | 43–54 | 47 | 47 | 77.5 |
| | | J | 43–49 | 46 | 83.8 | 43–48 | 46 | 47 | 82.9 |
| | Urine | V | 2–2 | 2 | 3.2 | 2–3 | 3 | 3 | 4.8 |
| | | C | 1–2 | 2 | 2.6 | 2–3 | 2 | 2 | 3.6 |
| | | J | 2–3 | 3 | 4.6 | 3–5 | 4 | 3 | 5.9 |
| | Retention | V | 6–11 | 8 | 15.3 | 4–10 | 7 | 7 | 12.0 |
| | | C | 8–14 | 10 | 17.4 | 7–16 | 11 | 11 | 18.9 |
| | | J | 4–9 | 6 | 11.6 | 4–10 | 7 | 6 | 11.2 |
| Phosphorus | Intake | V | 63–66 | 65 | .... | 64–68 | 66 | 66 | .... |
| | | C | 66–69 | 67 | .... | 66–69 | 68 | 68 | .... |
| | | J | 61–65 | 63 | .... | 62–65 | 63 | 64 | .... |
| | Feces | V | 21–23 | 22 | 34.0 | 21–26 | 23 | 24 | 36.4 |
| | | C | 20–22 | 21 | 30.9 | 19–25 | 22 | 22 | 32.7 |
| | | J | 20–25 | 23 | 37.2 | 22–27 | 24 | 25 | 38.7 |
| | Urine | V | 37–39 | 38 | 58.1 | 35–38 | 37 | 37 | 55.4 |
| | | C | 38–42 | 40 | 60.4 | 36–40 | 38 | 38 | 55.5 |
| | | J | 33–36 | 35 | 55.1 | 32–36 | 33 | 33 | 51.3 |
| | Retention | V | 4–6 | 5 | 7.9 | 2–8 | 6 | 5 | 8.2 |
| | | C | 3–10 | 6 | 8.7 | 6–10 | 8 | 8 | 11.8 |
| | | J | 2–8 | 5 | 7.7 | 4–7 | 6 | 6 | 10.0 |
| Nitrogen | Intake | V | 436–466 | 450 | .... | 603–627 | 618 | 617 | .... |
| | | C | 452–488 | 472 | .... | 619–643 | 635 | 632 | .... |
| | | J | 423–456 | 442 | .... | 579–692 | 595 | 591 | .... |
| | Feces | V | 32–35 | 33 | 7.3 | 36–48 | 41 | 43 | 7.0 |
| | | C | 44–46 | 45 | 9.6 | 41–56 | 49 | 50 | 7.9 |
| | | J | 52–59 | 56 | 12.5 | 56–61 | 59 | 59 | 9.9 |
| | Urine | V | 374–402 | 392 | 87.2 | 508–552 | 539 | 542 | 87.8 |
| | | C | 379–403 | 396 | 83.9 | 503–641 | 542 | 546 | 86.3 |
| | | J | 342–374 | 361 | 81.7 | 480–523 | 497 | 493 | 83.4 |
| | Retention | V | 13–27 | 25 | 5.5 | 23–78 | 38 | 32 | 5.2 |
| | | C | 14–47 | 31 | 6.5 | 27–84 | 44 | 36 | 5.8 |
| | | J | 10–34 | 25 | 5.8 | 16–64 | 39 | 39 | 6.7 |

[1] Percentage calculations were based on total daily figures in order to eliminate errors caused by rounding out the kilogram figures.

Although the actual changes in phosphorus values were very small, the 4-gm. protein diet slightly reduced the amount absorbed and thus reduced the amount of phosphorus available for use in the body. This was due to the fact that fecal outputs were 1 or 2 mg. per kilogram greater for each child and these higher values represented a larger percentage of the intake on the 4- than on the 3-gm. protein diet.

The increase in the protein content of the diet caused greater nitrogen absorption which represented no increase in the percentage of the intake for subject V but an increase of 2% of the intake for the other two children. The milligrams of nitrogen excreted in the feces increased for all children.

*Urinary excretion and retention.* The additional protein in the diet apparently did not affect the utilization of absorbed calcium. Although the average urine values per kilogram increased very slightly on the higher protein diet, the total values per day increased by a fairly large proportion of the urinary excretion. Since fecal values represented practically all of the calcium excreted, they obscured any changes in urine figures and made the retentions exactly the same on the two diets.

Although phosphorus retentions were only slightly higher on the 4- than on the 3-gm. protein diet, they represented a larger proportion of the intake values for each child and indicated that increased nitrogen produced a better utilization of phosphorus. On the 4-gm. protein diet the urinary phosphorus decreased more than the absorption figures for all except child V. The actual retentions in the body increased from 6 to 8 mg. per kilogram for subject C and from 5 to 6 mg. per kilogram for child J.

The higher protein diet caused an increase in the milligrams of nitrogen retained by all children. The percentage of the intake retained, however, was practically the same on the two diets.

## DISCUSSION

In both experiments, the differences in retention expressed in terms of retention ratios indicated that the metabolic

processes were not the same on the two levels of protein intake (table 3). The results of the two experiments showed that increased protein either from meat, milk and eggs or from egg white and gelatin produced similar changes in tissue growth.

TABLE 3

*Retention ratios.*[1]

| EXPERIMENT | PROTEIN IN DIET | CHILD | Ca·P | N:Ca | N:P | N:P — $\frac{Ca}{2 19}$[2] |
|---|---|---|---|---|---|---|
| | *gm.* | | | | | |
| 1 | 3 | B | 1.7:1 | 8.7:1 | 15.1:1 | 74:1 |
| | | D | 1.6:1 | 6.0:1 | 9.7:1 | 37:1 |
| | | Mean | 1.7:1 | 7.3:1 | 12.4:1 | 56:1 |
| | 4 | B | 1.1:1 | 10.0:1 | 10.8:1 | 21:1 |
| | | D | 1.0:1 | 8.4:1 | 8.3:1 | 15:1 |
| | | Mean | 1.0:1 | 9.2:1 | 9.5:1 | 18:1 |
| 2 | 3 | V | 1.7:1 | 2.9:1 | 4.8:1 | 21:1 |
| | | C | 1.7:1 | 3.0:1 | 5.3:1 | 27:1 |
| | | J | 1.3:1 | 4.0:1 | 5.3:1 | 13:1 |
| | | Mean | 1.6:1 | 3.3:1 | 5.1:1 | 20:1 |
| | 4 | V | 1.3:1 | 4.6:1 | 5.9:1 | 14:1 |
| | | C | 1.4:1 | 3.2:1 | 4.6:1 | 13:1 |
| | | J | 1.0:1 | 6.2:1 | 6.3:1 | 11:1 |
| | | Mean | 1.2:1 | 4.7:1 | 5.6:1 | 13:1 |

[1] On account of the small figures per kilogram of body weight and the errors involved, these ratios are calculated using the average daily retention values.

[2] $\frac{Ca}{2.19} = \frac{98\% \text{ calcium}}{2.15}$

The Ca:P retention ratios indicated that there was a difference in the relative growth of bone and soft tissue on the 3- and 4-gm. protein diets. From calculations based on chemical analyses of bone, muscle and other tissue, Stearns ('31) suggested that for infants a Ca:P retention ratio of from 1.5:1 to 1.6:1 represented the average relationship between bone and muscle tissue growth. A lower ratio indicated increased muscular development and a higher ratio indicated a greater growth of bone than muscle tissue. On the 3-gm. protein diet,

all children, with the exception of subject J, were probably producing a normal proportion of bone and muscle tissue since the retention ratios were either 1.6:1 or 1.7:1. With the additional protein in the diet, these ratios were reduced, ranging from 1.0:1 to 1.4:1. This showed an acceleration in the growth of soft tissue as compared to that of bone. Although subject J was apparently producing an excess of soft tissue growth on the 3-gm. protein diet, she, too, had an acceleration in the growth of that same type of tissue on the 4-gm. protein diet, because her retention ratio fell from 1.3:1 to 1.0:1.

Although all children grew at a faster rate on the 4- than on the 3-gm. protein diet (Hawks et al., '37, '38, and '40) the composition of the weight gains was probably not entirely the same for all children on the same diet nor for each child on the two different diets. If the increased weight gains had caused the acceleration of growth of bone tissue there should have been an increase in calcium retention, since the production of bones and teeth utilize from 97 to 98% of the absorbed calcium and the growth of other tissues requires only small amounts of this mineral. For both children in the first study and for child C in the second study, the higher protein diet did increase calcium retentions to a slight degree and thus these children may have had some acceleration of bone growth. For children V and J calcium retention did not increase and, therefore, the high protein diet probably had little if any effect on the bone growth of these children. Since the increase in bone growth on the high protein diet was probably small, soft tissue must have composed a large part of the increased weight gains. The higher nitrogen retentions on the 4-gm. protein diet substantiated this fact. Water storage may have produced some of the extra weight gains, because there was an increase in sodium and chlorine retentions on the higher protein diet and these minerals are associated with fluid tissues in the body (Hawks, Bray, Hartt, Whittemore and Dye, in press). Furthermore fat deposits

may have caused part of the increased weight gains and some phosphorus may have been deposited with this tissue.

The relative growth of bone and protein tissue can also be expressed by a ratio between nitrogen and calcium retentions provided one assumes that all of the nitrogen is utilized in production of protein tissue, and all of the calcium in bony tissue. The change to the 4-gm. protein diet caused an increase in the N : Ca retention ratios for all children, indicating that nitrogen storage increased to a greater extent than that of calcium, and that in all probability protein tissue increased at a faster rate than did bone tissue. In the first study the ratios changed from a mean of 7.3 : 1 on the 3- to a mean of 9.2 : 1 on the 4-gm. protein diet, and in the second study the corresponding changes were from 3.3 : 1 to 4.7 : 1 (table 3).

The N : P retention ratios gave further evidence that all five children were not producing the same type of body tissue. The lower ratios on the higher protein diet showed increased retentions of phosphorus in relation to nitrogen for children B, D and C. This may be explained by the fact that additional phosphorus was probably deposited with the extra calcium which these children stored. There was an increase in the ratios for subjects V and J who did not store additional calcium.

It would be possible to signify differences in the type of soft tissue growth by a ratio between nitrogen and the amount of phosphorus used in excess of that deposited in bones. Since bone contains 2.15 parts of calcium to 1 part of phosphorus (Shohl, '39), it is possible to estimate the amount of phosphorus deposited in the skeleton if one assumes that 98% of the calcium is deposited therein (Stearns, '31). The amount of phosphorus retained in excess of that required for bone growth may then be expressed by the relation, P retained in soft tissues = total P retained $- \frac{98\% \text{ of the calcium}}{2.15}$ . This retention ratio then becomes N : P $- \frac{Ca}{2.19}$.

The ratios between nitrogen and this new value for phosphorus indicated that on the lower protein diet the children were producing tissues which did not have a composition

similar to that of body tissue as a whole. With the exception of child J, the ratios were all above 17:1 which Stearns ('31) said approximated that for all body tissue other than bone. This suggests that the children were at that time manufacturing tissue of some particular type high in nitrogen. In blood, for example, the ratio is 53:1. It is also possible that some of that nitrogen may have been used to build up a reserve in the body and that all of it may not have been laid down in conjunction with phosphorus in muscle tissue.

The reduction of these ratios on the high protein diet may indicate that muscle was the dominant type of tissue manufactured, since it has an approximate N: non-osseous P ratio of 15:1. Calcium utilization was practically the same on the two diets, thus nitrogen intake probably had little influence on bone growth.

### SUMMARY

1. Five pre-school children received, during 15- to 24-day periods, diets containing first, 3 and then 4 gm. of protein per kilogram of body weight.

2. For two children whole egg, meat and milk increased the nitrogen content of the diet while for the others egg white and gelatin were used.

3. The higher protein diet had no effect on calcium absorption or retention. It caused a decrease in the absorption of phosphorus but there was a better utilization of the amount available. There was an increase in both nitrogen absorption and retention.

4. Weight gains were greater on the 4- than on the 3-gm. protein diet and represented different types of tissue growth. Decided increases in nitrogen retentions and significant changes in retention ratios for Ca:P, N:Ca, N:total P and N: non-osseous P, suggested that muscle and other soft tissue accounted for the greater proportion of the increased weight gains. There may have been additional bone growth in three children who had increased calcium retentions.

## LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1925 Official and tentative methods of analysis. The Association, Washington, D. C.

HAWKS, J. E., M. DYE AND M. M. BRAY 1937 An improved technic for metabolism studies in pre-school children with a statistical determination of its reliability. J. Nutrition, vol. 13, p. 51.

HAWKS, J. E., M. M. BRAY AND M. DYE 1938 The influence of diet on the nitrogen balances of pre-school children. J. Nutrition, vol. 15, p. 125.

HAWKS, J. E., J. M. VOORHEES, M. M. BRAY AND M. DYE 1940 The influence of the nitrogen content of the diet on the calorie balances of pre-school children. J. Nutrition, vol. 19, p. 77.

HAWKS, J. E., M. M. BRAY, S. HARTT, M. B. WHITTEMORE AND M. DYE Potassium, sodium and chlorine balances of pre-school children receiving medium and high protein diets. (In press.)

KRAMER, B., AND J. HOWLAND 1926 The quantitative estimation of calcium, magnesium, phosphate, and carbonate in bone. J. Biol. Chem., vol. 68, p. 711.

PETERS, J. P., AND D. D. VAN SLYKE 1932 Quantitative clinical chemistry. Vol. II, Methods, p. 850. The Williams and Wilkins Co., Baltimore.

SHOHL, A. T. 1939 Mineral metabolism. Am. Chem. Soc. Monograph No. 82. Reinhold Publishing Corp.

SHERMAN, H. C. 1941 Chemistry of food and nutrition, 6th edition. The Macmillan Company, New York.

STEARNS, G. 1931 The significance of the retention ratio of calcium: phosphorus in infants and in children. Am. J. Dis. Child., vol. 42, p. 749.

# THE EFFECT OF THE LEVEL OF PROTEIN INTAKE UPON THE URINARY EXCRETION OF RIBOFLAVIN AND NICOTINIC ACID IN DOGS AND RATS [1]

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TWO FIGURES

There is evidence to indicate a relationship between protein metabolism and the vitamins of the B-complex. An increase in the protein content of the diet without a corresponding increase in B-complex supplement results in poor growth of rats (Hartwell, '25; Reader and Drummond, '25). The development of a B-avitaminosis in rats is accelerated by diets high in protein (Tscherkes, '26). In self-selection feeding experiments rats eat more protein when given the B-vitamins (Richter and Barelare, '39). The dermatitis produced by a deficiency of pyridoxine occurs more readily on a diet containing 30% casein than on a diet containing lower levels of protein (Conger and Elvehjem, '41). Retention of protein by chicks on diets deficient in riboflavin is less than that of pair-fed controls (Kleiber and Jukes, '42). In growing rats riboflavin was found to have a profound effect on the utilization of food for the synthesis of tissue fat and protein (Sure and Dichek, '41).

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In the present work, the relationship of protein metabolism to the vitamins, riboflavin and nicotinic acid, was studied in dogs and rats by measuring the urinary excretion of the vitamins as a function of protein intake. In dogs the excretion of thiamine and pyridoxine was also measured.

<center>MATERIALS AND METHODS</center>

Young and adult dogs, maintained in individual cages, and young and adult rats, maintained in groups of four to six, were fed diets containing mixtures of purified casein,[3] beef suet, cornstarch, sucrose, salt mixture, cod liver oil, and dried brewer's yeast.[4] The fats and a suspension of casein and yeast were added to a hot mixture of salts, sucrose, and starch that had been cooked about 45 minutes. The final mixture was cooled and kept in the refrigerator. The diets were to provide three different levels of protein, designed and fed so as to provide approximately the same caloric and vitamin intake per unit of body weight. Each dog was given daily 175 gm. (160 cal.) of cooked food per kilo of body weight. Compensating changes in carbohydrate or fat, or both, were made for changes in protein intake. The composition of the diets used is given in table 1. The low protein diet contained 2.6%, the medium protein diet 17.6% and the high protein diet 41% of the total calories as protein.

Each animal, or group, was maintained at least once for a period of 1 to 2 weeks on each diet, and in the case of the dogs, was given a test dose of riboflavin, nicotinic acid, pyridoxine, and thiamine at the end of each period. The daily food intake was measured, and the animals were weighed every second day.

Urines were collected daily in dark bottles containing toluene and sufficient hydrochloric acid to give a final pH of about 2. Analyses were made on daily or combined samples of urine. Riboflavin was determined fluorometrically (Ferrebee, '40), total nicotinic acid (trigonelline and acid-hydro-

[3] Labco.
[4] Fleischmann.

lyzable derivatives) chemically (Perlzweig, Levy and Sarett, '40), thiamine by its effect on yeast fermentation (Atkin, Schultz and Frey, '39), and nitrogen by nesslerization after digestion with sulfuric acid and hydrogen peroxide. The method employed to measure pyridoxine (Scudi, Buhs and Hood, '42; Bird, Vandenbelt and Emmett, '42) permitted only the analysis of the response to the test dose.

The details of experiments that differ from those outlined above are given in the appropriate place.

TABLE 1

*Composition of experimental diets of varying protein content.*

| INGREDIENT | FOR DOGS | | | FOR RATS | | |
|---|---|---|---|---|---|---|
| | Low | Medium | High | Low | Medium | High |
| | gm. | gm. | gm. | gm. | gm. | gm. |
| Purified casein (Labco) | 1 | 18 | 45 | 1 | 18 | 45 |
| Cornstarch | 40 | 40 | 40 | 59 | 60 | 60 |
| Sucrose | 19(60)[1] | 27 (2)[1] | 20 | .. | 7 | .. |
| Beef suet | 21 (2)[1] | 10(21)[1] | 1 | 21 | 10 | 1 |
| Cod liver oil | 2 | 2 | 2 | 2 | 2 | 2 |
| B.D.H. salt mixture | 3 | 3 | 3 | 3 | 3 | 3 |
| Dried brewer's yeast [2] (Fleischmann) | 4.5 | 4.5 | 4.5 | 4 | 4 | 4 |
| Water added to give 500 gm. of cooked diet. | | | | | | |

[1] Fat and carbohydrate contents of the diets were reversed in some experiments to show that the effects obtained were not due to the particular concentrations of these substances.

[2] The yeast contained about 25 μg. of thiamine, 50 μg. of riboflavin and 520 μg. of nicotinic acid per gram.

### EXPERIMENTAL

*Effect of protein intake on the urinary excretion of riboflavin, nicotinic acid, and thiamine.* The data obtained on three growing dogs (G, H, and I) and two adult dogs (J and K), are given in table 2. The data show that the excretion of riboflavin and total nicotinic acid was highest during the periods of low protein intake. The increase in protein intake corresponding to the change from the medium to the high

protein diet had practically no effect on the excretion of riboflavin, but decreased the excretion of nicotinic acid slightly. The thiamine excretion during the periods indicated by table 2 was measured, but since there was no correlation with the

TABLE 2

*The average daily intake and excretion of nitrogen, riboflavin and nicotinic acid by five dogs on low, medium and high protein diets, and the per cent excretion of test doses of the vitamins after periods on these diets.*

| DOG | DIET | NO. OF DAYS | CHANGE IN BODY WEIGHT | NITROGEN | | RIBOFLAVIN | | NICOTINIC ACID | | EXCRETION OF TEST DOSE AFTER EACH PERIOD [2] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Intake | Urinary excretion | Intake | Urinary excretion | Intake | Urinary excretion [1] | Riboflavin | Nicotinic acid | Pyridoxine |
| | | | kg. | gm. | gm. | μg. | μg. | mg. | mg. | % | % | % |
| G (3.2 kg.) | High | 14 | + 0.4 | 7.1 | 2.9 | 209 | 71 | 2.2 | 0.6 | | | |
| | Low | 11 | − 0.2 | 0.6 | 0.8 | 277 | 401 | 2.9 | 1.5 | 78 | 35 | 36 |
| | Med. | 6 | + 0.2 | 4.1 | 1.5 | 287 | 71 | 3.0 | 1.2 | 14 | 32 | 24 |
| | Low | 6 | − 0.1 | 0.4 | 0.7 | 197 | 350 | 2.0 | 1.5 | 62 | 28 | 31 |
| | High | 6 | + 0.3 | 7.7 | 3.1 | 230 | 85 | 2.7 | 0.9 | 6 | 35 | 20 |
| H (4.2 kg.) | Low | 14 | − 0.1 | 0.6 | 0.7 | 300 | 212 | 3.2 | 2.3 | | | |
| | Med. | 11 | + 0.5 | 4.7 | 1.6 | 324 | 64 | 3.4 | 1.7 | 32 | 50 | 31 |
| | Med. | 6 | + 0.4 | 5.6 | 1.8 | 387 | 70 | 4.1 | 1.2 | 32 | 50 | 23 |
| | Low | 6 | + 0.2 | 0.9 | 1.0 | 428 | 485 | 4.4 | 2.3 | 68 | 75 | 24 |
| | High | 6 | + 0.6 | 10.2 | 3.2 | 302 | 73 | 3.1 | 1.9 | 3 | 32 | 20 |
| I (2.8 kg.) | Med. | 14 | + 0.2 | 2.8 | 1.0 | 195 | 64 | 2.0 | 0.7 | | | |
| | High | 11 | + 0.7 | 7.2 | 2.2 | 222 | 83 | 2.3 | 1.0 | 0 | 24 | 13 |
| | Med. | 6 | + 0.2 | 4.1 | 1.6 | 283 | 65 | 3.0 | 1.0 | 31 | 47 | 20 |
| | Low | 6 | + 0.1 | 0.6 | 0.7 | 320 | 405 | 3.3 | 1.3 | 74 | 51 | 19 |
| | High | 6 | + 0.7 | 8.5 | 2.4 | 250 | 83 | 2.6 | 1.3 | 3 | 37 | 18 |
| J (5.9 kg.) | High | 9 | + 0.1 | 4.8 | 3.5 | 147 | 65 | 1.5 | 1.0 | 31 | 68 | 57 |
| | Low | 9 | − 0.2 | 0.4 | 1.4 | 334 | 260 | 3.5 | 2.6 | 75 | 97 | 78 |
| K (7.1 kg.) | Med. | 6 | 0 | 2.2 | 1.4 | 164 | 64 | 1.7 | 0.9 | 32 | 60 | 53 |
| | Low | 10 | − 0.1 | 0.2 | 1.1 | 312 | 129 | 3.2 | 1.7 | 63 | 81 | 61 |
| | High | 9 | + 0.2 | 5.2 | 3.3 | 160 | 57 | 1.7 | 0.7 | 29 | 65 | 47 |
| | Low | 9 | − 0.2 | 0.3 | 1.3 | 270 | 184 | 2.8 | 1.4 | 100 | 69 | .. |

[1] Nicotinic acid excretion represents total urinary trigonelline and acid hydrolyzable nicotinic acid derivatives.

[2] The test doses were given subcutaneously and contained 220 μg. riboflavin, 2.2 mg. nicotinamide, 2.2 mg. pyridoxine and 110 μg. thiamine per kilo body weight. The figures given represent the per cent of the dose excreted after subtraction of the value for the preceding day.

protein intake the data are not given. The effects of reciprocal changes in carbohydrate and fat content of low and medium protein diets (table 1) were studied during periods not shown in table 2. The changes did not affect the excretion of riboflavin or total nicotinic acid.

Data on dog G are given in figure 1 showing the changes in riboflavin and nicotinic acid excretion during the periods indicated in table 2. The data in figure 1 and similar data on the other dogs indicate that the change in riboflavin excretion
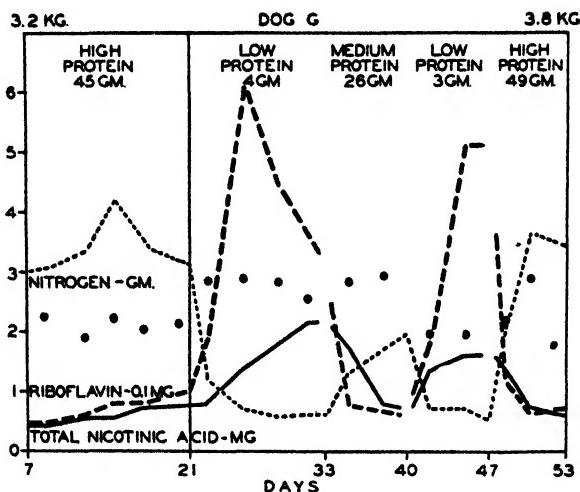


Fig. 1  The urinary excretion of nitrogen, riboflavin, and nicotinic acid by dog G on low, medium and high protein diets. The single black dots represent the riboflavin intake.

produced by the transfer from the medium or high protein diet to the low one, or the converse, was comparatively rapid, and that a maximum excretion on the low diet was reached in 3 to 6 days. The changes in nicotinic acid excretion were slower than those in riboflavin excretion. The excretion of nicotinic acid on the low protein diet continued to increase during 6 to 14 days of those periods, except for the first period of dog H where the excretion was approximately constant from the seventh to the fourteenth day.

As indicated by the data in figure 2 the effect of the protein content of the diet on the urinary excretion of riboflavin by young and adult rats was similar to that obtained in dogs. In rats, however, the increase in protein intake corresponding to the change from the medium to the high protein diet lowered the excretion of riboflavin. The effect of the diets on the excretion of nicotinic acid by rats differed from the effect obtained in dogs. As shown in figure 2 the change from the medium
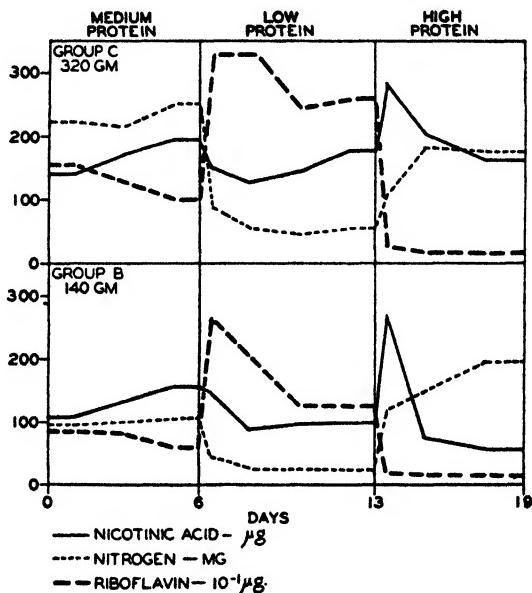


Fig. 2   The urinary excretion of nitrogen, riboflavin, and nicotinic acid per rat, by young and adult male rats on low, medium and high protein diets.

to the low protein diet resulted in an initial decrease in excretion. The change from the low to the high protein diet resulted in an initial increase in excretion which is followed by a decrease. Changes in the excretion of nicotinic acid by rats similar to those shown in figure 2 have been obtained by feeding protein and other nitrogenous compounds (Huff and Perlzweig, '42). The difference between the effect in dogs and in rats may be due to the fact that rats synthesize nicotinic acid (Huff and Perlzweig, '42; Dann, '41).

The low urinary excretion of riboflavin and of nicotinic acid on the medium and high protein diets was associated with an increased excretion of nitrogen, a positive nitrogen balance, and a gain in weight. The high excretion of riboflavin and of nicotinic acid on the low protein diets was associated with a decreased excretion of nitrogen, a negative nitrogen balance, and occasionally with a loss of weight.

The low urinary excretion of the vitamins on the medium and high protein diets may be due either to the catabolism of protein, which leads to the increase in urinary nitrogen, or to the anabolism of protein which leads to the retention of protein nitrogen, or both. The following experiments suggest that the low excretion is not due to the catabolism of protein.

*Effect of protein intake on the response to test doses of riboflavin, nicotinic acid, pyridoxine and thiamine.* A mixture containing 220 µg. of riboflavin, 2.2 mg. of nicotinamide, 110 µg. of thiamine, and 2.2 mg. of pyridoxine [5] per kilo of body weight was injected into the dogs subcutaneously on the last day of each period indicated in table 2. The differences between the excretions on the preceding day and on the day of the test dose are reported in table 2 as the per cent of the test dose excreted. Since the results of the test dose in the case of thiamine were not affected by the protein intake the data are omitted.

The per cent of the test dose of riboflavin excreted by all dogs was highest on the low protein diet and lowest on the high protein diet. Where a difference in response to the test dose of nicotinic acid was obtained (dogs H, I, J, and K) the per cent of the test dose excreted was highest on the low protein diet. Where a difference in response to the test dose of pyridoxine was obtained (dogs G, J, and K) the per cent of the test dose excreted was also highest on the low protein diet.

If the differences between the responses to the test doses on the various diets represent differences in retention, the data

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[5] These synthetic vitamins were generously supplied by Merck and Company, Rahway, New Jersey.

for riboflavin indicate a greater retention as the protein content of the diet is increased. The data for nicotinic acid and pyridoxine suggest a similar relationship between retention and protein intake.

*Effect of the replacement of casein by glycine on the urinary excretion of riboflavin and nicotinic acid by dogs.* Three young dogs were maintained for several days on the medium protein diet, and then on a similar diet in which the casein was replaced by glycine in terms of nitrogen equivalents. The data obtained on the three dogs were sufficiently uniform to permit consideration of average values. The averages are presented in table 3. The increase in excretion of riboflavin produced by

TABLE 3

*The effect of substitution of glycine for casein in the diet of three dogs, L, M and O, upon the urinary excretion of nitrogen, riboflavin and nicotinic acid.*

| DIET | DAYS | AVERAGE WEIGHT | NITROGEN | | RIBOFLAVIN | | NICOTINIC ACID | |
|---|---|---|---|---|---|---|---|---|
| | | | Intake | Urinary excretion | Intake | Urinary excretion | Intake | Urinary excretion |
| | | *kg.* | *gm.* | *gm.* | *μg.* | *ug.* | *mg.* | *mg.* |
| Medium | | 2.51 | 2.8 | 0.8 | 190 | 36 | 2.1 | 0.4 |
| Medium diet | 1 | 2.51 | 3.2 | 2.8 | 190 | 49 | 2.1 | 1.3 |
| with glycine | 2 + 3 | 2.54 | 3.2 | 2.7 | 190 | 69 | 2.1 | 1.5 |
| substituted | 4 | 2.54 | 3.2 | 2.8 | 190 | 119 | 2.1 | 2.0 |
| for casein | 5 | 2.52 | 3.2 | 2.5 | 190 | 165 | 2.1 | 1.9 |
| | 6 | 2.48 | 3.1 | 2.4 | 187 | 146 | 2.1 | 2.1 |

the change from casein to glycine was similar to that obtained in the other dogs by the change from the medium to the low protein diet; however, in the change to glycine the increase in excretion of riboflavin occurred somewhat more slowly and the increase in excretion of nicotinic acid somewhat more rapidly than in the change to the low protein diet.

The fact that the excretion of riboflavin and nicotinic acid increased during a period of active amino acid catabolism suggests that the decrease in excretion of riboflavin and nicotinic acid produced by an increased protein intake (cf. table ·2) was not due to the catabolic processes that led to the increase in urinary nitrogen.

*Effect of starvation on the urinary excretion of riboflavin and nicotinic acid by dogs.* The data in table 4 show that the urinary excretion of riboflavin and nicotinic acid increased during a period of starvation, which represented a period of

TABLE 4

*The effect of starvation upon the daily urinary excretion of nitrogen, riboflavin and nicotinic acid by three young dogs.*

| DIET | DAYS | URINARY NITROGEN | | | URINARY RIBOFLAVIN | | | URINARY TOTAL NICOTINIC ACID | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | L 3.2 kg. | M 2.6 kg. | O 3.5 kg. | L | M | O | L | M | O |
| Medium | | *gm.* 1.0 | *gm.* 0.5 | *gm.* 1.0 | *μg* 36 | *μg.* 25 | *μg.* 40 | *mg.* 0.6 | *mg.* 0.4 | *mg.* 0.6 |
| Fasting | 1 + 2 | 1.2 | 0.7 | 1.3 | 43 | 29 | 43 | 2.1 | 0.9 | 1.0 |
| | 3 + 4 | 1.4 | 0.9 | 1.4 | 83 | 48 | 56 | 2.3 | 1.5 | 1.4 |
| | 5 + 6 | 1.5 | 0.6 | 1.3 | 195 | 78 | 116 | 3.6 | 1.7 | 3.8 |
| | 7 | 1.0 | 0.8 | 1.3 | 124 | 130 | 250 | 2.5 | 1.9 | 2.8 |
| 50–120 gm. of corn-starch and sucrose per day | 8 | 1.1 | 0.8 | 0.9 | 108 | 105 | 105 | 2.6 | 1.7 | 1.2 |
| | 9 + 10 | 0.4 | 0.2 | 0.3 | 63 | 29 | 52 | 0.7 | 0.3 | 0.4 |
| Medium [1] | | | 1.2 | 1.6 | | 33 | 38 | | 0.5 | 1.1 |
| Fasting plus a vitamin capsule [2] | 1 + 2 | | 0.9 | 1.2 | | 41 | 38 | | 1.9 | 5.1 |
| | 3 + 4 | | 1.0 | 1.5 | | 130 | 120 | | 3.7 | 3.8 |
| | 5 + 6 | | 1.5 | 1.5 | | 550 | 460 | | 4.2 | 3.8 |
| | 7 + 8 | | 1.4 | 1.5 | | 597 | 525 | | 4.1 | 2.9 |

[1] Body weights of M and O at the beginning of this experiment were 3.4 kg. and 4.4 kg., respectively.

[2] Vitamin capsule (Lederle) was a concentrated liver extract and contained 1.5 mg. of nicotinamide and 375 μg. of riboflavin.

protein metabolism that was catabolic.[6] The ingestion of carbohydrate after the period of starvation decreased the catabolism of protein and the excretion of the vitamins. These experiments again suggest that the decrease in excretion of riboflavin and nicotinic acid accompanying an increase in pro-

[6] Similar increases in riboflavin excretion were also found in eleven normal human subjects during 3 to 6-day periods of fasting.

tein intake is due to an anabolic process and is not due to participation in protein catabolism.

Dogs M and O were subjected to another period of fasting a few weeks later, during which each received a daily oral dose of a liver concentrate[7] containing 375 μg. of riboflavin and 1.5 mg. of nicotinamide. The vitamin intake had no obvious effect on the nitrogen excretion. A significant increase in the excretion of riboflavin above that obtained during the first period of fasting when no vitamins were given did not occur until the third or fourth day. An increase in nicotinic acid excretion above that obtained during the first period of fasting was observed as early as the first day. The degree to which the vitamin concentration of the tissues of the animal approaches a maximum level and the efficiency of the mechanisms for conserving each of the vitamins may account for the delay in the excretion of the ingested riboflavin and the prompt appearance of the ingested nicotinic acid. These factors may also determine in part the rate and extent of change in excretion produced by the different diets.

### DISCUSSION

The preceding experiments show that an increase in the protein content of the diet, associated with a positive nitrogen balance, produced a decrease in the urinary excretion of nicotinic acid by dogs and of riboflavin by both dogs and rats. The decrease in urinary excretion of the vitamins was probably due, in part, to a retention of the vitamins, since a decrease in the protein content of the diet, associated with a negative nitrogen balance, produced an increase in the urinary excretion of the vitamins. The apparent retention of the vitamins on the high protein diet was probably not due to the increased protein catabolism, since the catabolism during starvation and during the period of glycine ingestion was accompanied by an increased excretion of the vitamins. If the retention of the vitamins during the period of high protein ingestion was not due to protein catabolism, it is conceivable

[7] Lederle.

that the retention was due mainly to protein anabolism. Whether there is actual retention, and whether it is due to protein anabolism, are being investigated by complete balance experiments including tissue analyses. That riboflavin is concerned with protein anabolism is shown (Kleiber and Jukes, '42) by the fact that the protein content of chicks deficient in riboflavin is less than that of pair-fed controls. The experiments described also suggest that a similar relationship may exist between protein anabolism and nicotinic acid and pyridoxine.

## SUMMARY

The urinary excretions of nicotinic acid by dogs and of riboflavin by both dogs and rats bear an inverse relationship to the level of protein intake.

Riboflavin and nicotinic acid are inferred to be concerned in protein anabolism.

### LITERATURE CITED

ATKIN, L., A. S. SCHULTZ AND C. N. FREY 1939 Ultramicrodetermination of thiamine by the fermentation method. J. Biol. Chem., vol. 129, p. 471.

BIRD, O. D., J. M. VANDENBELT AND A. D. EMMETT 1942 Adaptation of the Scudi colorimetric method for pyridoxine. J. Biol. Chem., vol. 142, p. 317.

CONGER, T. W., AND C. A. ELVEHJEM 1941 The biological estimation of pyridoxine. J. Biol. Chem., vol. 138, p. 555.

DANN, W. J. 1941 The synthesis of nicotinic acid by the rat. J. Biol. Chem., vol. 141, p. 803.

FERREBEE, J. W. 1940 The urinary excretion of riboflavin. Fluorometric methods for its estimation. J. Clin. Invest., vol. 19, p. 251.

HARTWELL, G. A. 1925 Mammary secretion in rats. VI. Vitamin B and the lactating rat's diet. Biochem. J., vol. 19, p. 1075.

HUFF, J. W., AND W. A. PERLZWEIG 1942 Studies in nicotinic acid metabolism. III. Metabolism and synthesis of nicotinic acid in the rat. J. Biol. Chem., vol. 142, p. 401.

KLEIBER, M., AND T. H. JUKES 1942 Metabolism and food utilization of riboflavin-deficient chicks. Proc. Soc. Exp. Biol. Med., vol. 49, p. 34.

PERLZWEIG, W. A., E. D. LEVY AND H. P. SARETT 1940 Nicotinic acid derivatives in human urine and their determination. J. Biol. Chem., vol. 136, p. 729.

READER, V., AND J. C. DRUMMOND 1925 Relation between vitamin B and protein in the diet of growing rats. Biochem. J., vol. 20, p. 1256.

RICHTER, C. P., AND B. BARELARE, JR.   1939   Further observations on the carbo-
        hydrate, fat and protein appetite of vitamin B deficient rats.   Am. J.
        Physiol., vol. 127, p. 199.
SCUDI, J. V., R. P. BUHS AND D. B. HOOD   1942   The metabolism of vitamin $B_6$.
        J. Biol. Chem., vol. 142, p. 323.
SURE, B., AND M. DICHEK   1941   Riboflavin as a factor in economy of food utiliza-
        tion.   J. Nutrition, vol. 21, p. 453.
TSCHERKES, L. A.   1926   Studien über B-avitaminose 1.   Biochem. Z., vol. 167,
        p. 203.

ERRATUM

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M. E. Yarbrough and W. J. Dann

In table 2, page 601, the third entry should read:

Caveness et al. ('41)     Mean 138              Mean 62
                                 stand. dev. 62        stand. dev. 17

# THE EFFECT OF GLUCOSE AND SUCROSE ON THE RESPIRATORY QUOTIENT AND MUSCULAR EFFICIENCY OF EXERCISE

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TWO FIGURES

The controversial question of just how fats and carbohydrates are utilized for muscular energy has been studied from many angles. One of the most important of these is the interpretation of the respiratory quotients obtained during exercise. The specific effects on the respiratory quotient following the ingestion of various sugars were first studied by Carpenter and his associates (Carpenter and Fox, '31 a, b; Carpenter and Lee, '38). The resting R.Q. was increased by fructose to a much greater extent than by glucose, even after a correction was made for the greater lactic acid accumulation caused by fructose. It was suggested that the higher R.Q. was due to the conversion of fructose to fat. The total work R.Q. was also increased more by fructose than by glucose. However, more glucose than fructose was used in the excess metabolism occasioned by work. The apparent increment in the work R.Q. caused by fructose was probably due only to this sugar's conversion to fat. Carpenter and his associates were able to find no significant difference in the efficiency of the work after the ingestion of sugars.

Haggard and Greenberg ('35) obtained R.Q. values after the ingestion of sucrose and glucose which were very similar

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to those of Carpenter; the greater increase found for sucrose was probably due to its fructose component. In contrast to Carpenter's findings, however, they reported that the mechanical efficiency of exercise paralleled very closely the changes in the R.Q. after each sugar.

Bachmann, Haldi and assistants ('37; Haldi and Bachmann, '37; Haldi, Bachmann and Ensor, '38) confirmed the work of Carpenter and his associates in every respect. They were unable to find any correlation between efficiency and R.Q.

In the work reported herewith, the effects of glucose and sucrose on the resting and work R.Q.'s and on the muscular efficiencies are found to confirm the conclusions of Carpenter. In addition, an interesting relationship between resting and work R.Q.'s is described.

<div align="center">METHODS</div>

The present work was done on a Krogh bicycle ergometer. The subject pedalled at a uniform rate for 10 minutes, accomplishing 15 kilo-calories of work. The respiratory exchange was obtained with a Benedict Universal machine which was carefully tested for leaks before every run and checked for accuracy at intervals with an alcohol flame. The alcohol R.Q.'s ranged from 0.67 to 0.68 (theoretical — 0.667). Most of the experiments were done on one young male student about 7 hours after breakfast.[1] Data for an initial rest period of 7 minutes were obtained while he sat on the bicycle. After the subsequent 10-minute work period, he stopped pedalling but continued to breathe into the system for 15 to 20 minutes until recovery was complete, as indicated by the return of the alveolar $CO_2$ concentration, pulse and respiration rates to rest levels. Fifty grams (unless otherwise indicated) of

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[1] No attempt was made to control the diet, since one of the points of interest in this study was the relationship of the muscular efficiency to different R.Q.'s. The erratic eating habits of this subject (his breakfasts, which he occasionally missed completely, varied from sugar cookies to the conventional large breakfast) were reflected in the great variability of the baseline R.Q.'s. Notes made at the time showed that the R.Q.'s varied in the directions expected from the quality and quantity of his breakfasts.

sucrose or an equicaloric amount of glucose in 400 cc. of water containing the juice of one lemon were then ingested. After 30 minutes he returned to the bicycle, sat through another rest period, and then was observed through a period of work and recovery.

Nitrogen determinations in the urine were omitted as they were shown in preliminary experiments to alter the R.Q.'s only in the third decimal place.

The glucose was given in the form of a spray-dried corn syrup [2] and a more hydrolyzed form of corn syrup.[3]

### RESULTS

#### *The respiratory quotient*

The variation in the baseline R.Q.'s (6–8 hours after breakfast) from 0.69 to 1.00 shows that the subjects were not in a uniform state; however, there is presented the opportunity to study the metabolism of work over a wide range of rest R.Q. values. The average R.Q. for 43 baseline rest periods is 0.87; for the total work and recovery, 0.88; and for the excess metabolism due to the work alone, 0.89. In figure 1, the resting R.Q.'s are arranged in numerical order, in this way falling along a regularly rising curve from the left to the right of the figure. The work and recovery R.Q.'s lie in a narrow horizontal band around the average value of 0.88. It will be seen that the work and recovery R.Q.'s are higher than their corresponding rest R.Q.'s when the latter are less than 0.88. Work and recovery R.Q.'s corresponding to rest R.Q.'s of 0.88 and 0.89 are numerically identical, and above this they are lower than their rest R.Q.'s. The excess R.Q.'s vary from their corresponding rest R.Q.'s in a similar manner, but to a greater extent, thus falling along a descending curve as the rest R.Q.'s increase. Again, in the center of the figure where the rest R.Q.'s are 0.88 and 0.89, the excess values are very close to these numbers. The

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[2] This is not all preformed glucose, but quickly yields only glucose in digestion.
[3] "Sweetose" supplied by the Staley Manufacturing Company.

greatest differences occur at the two extremes of the rest R.Q. scale.

The results obtained 30 minutes after the ingestion of the sugars are similarly arranged in figure 2. The average value for the rest R.Q. is 0.93; for the work and recovery R.Q., 0.92; and for the excess R.Q., 0.91. As in figure 1, the R.Q. values fall into a similar pattern, in which the same relations exist between rest, work and recovery, and excess



FIG.1                    FIG.2
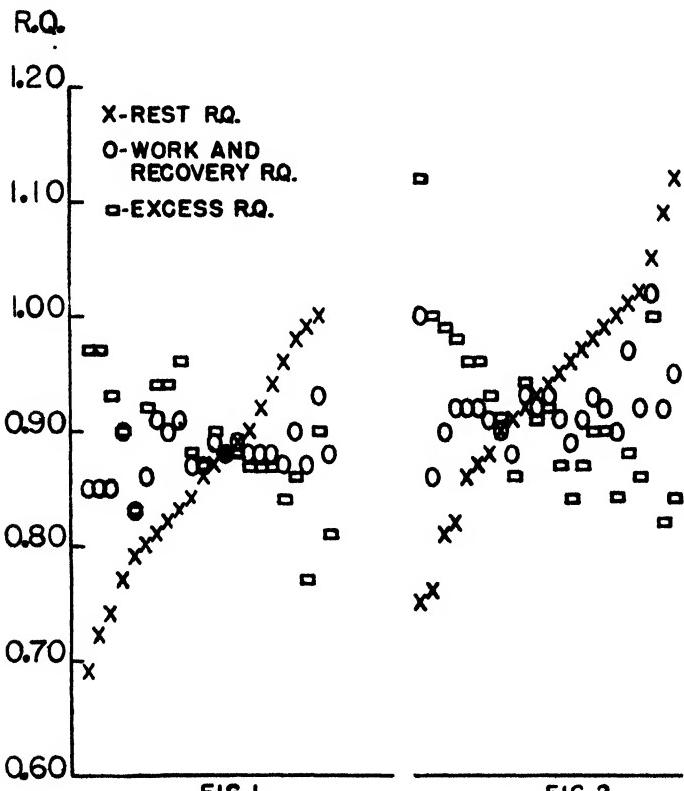
R.Q.'s before sugar.          R.Q.'s after sugar.

The averages of the work and recovery and excess R.Q.'s lie in a vertical line with their corresponding rest R.Q.'s. R.Q.'s corresponding to rest values from 0.80 to 0.96 in figure 1 and from 0.86 to 0.99 in figure 2 are averages of from 2 to 4 runs. Some values obtained in other experiments not included in the tables have been added to this chart.

R.Q.'s as were found in the baseline experiments. As expected, the pattern is placed at a higher level in the R.Q. scale. In this case, the three R.Q.'s coincide at rest levels of 0.92–0.93.

## The effect of different sugars on the R.Q.

Sucrose (table 1) increased the rest R.Q. from an average of 0.84 to 0.92 — a difference of 0.08; whereas, glucose (table 2) elicited a rise of only 0.01. To determine the amounts of

TABLE 1

*The R.Q. and per cent net efficiency before and after sucrose.*

| SUBJECT | DATE | BEFORE SUCROSE | | | | AFTER 50 GM SUCROSE | | | |
|---------|------|------|------------------------|--------|---------------------------|------|------------------------|--------|---------------------------|
| | | Rest | R.Q. Work and recovery | Excess | Per cent net efficiency | Rest | R.Q. Work and recovery | Excess | Per cent net efficiency |
| S.W. | 12/13 | .90 | .82 | .76 | 19.77 | .91 | .86 | .82 | 24.47 |
| S.W. | 12/17 | .94 | .81 | .79 | 19.13 | .99 | .93 | .88 | 20.34 |
| G.D. | 2/14 | .94 | .91 | .88 | 19.87 | .98 | .93 | .89 | 19.17 |
| G.D. | 2/19 | .72 | .85 | .95 | 20.08 | .76 | .88 | 1.00 | 21.17 |
| G.D. | 2/29 | | | | | .81 | .90 | .99 | 21.32 |
| G.D. | 2/21 | .74 | .86 | .93 | 19.31 | .93 | .90 | .87 | 19.40 |
| G.D. | 2/22 | .72 | .87 | 1.01 | 22.87 | .93 | .94 | .94 | 20.90 |
| G.D. | 2/26 | .81 | .96 | 1.04 | 20.88 | .87 | .95 | 1.03 | 21.17 |
| R.K. | 2/27 | .94 | .91 | .89 | 20.61 | .92 | .93 | .93 | 21.81 |
| R.K. | 2/28 | .84 | .87 | .88 | 19.47 | .93 | .91 | .91 | 20.45 |
| R.K. | 2/29 | .88 | .84 | .81 | 19.73 | 1.04 | 1.02 | 1.00 | 21.59 |
| Average | | .84 | .87 | .89 | 20.18 | .92 | .92 | .93 | 21.07 |
| Net increase ("after" minus "before sugar") | | | | | | .08 | .05 | .04 | .89 |
| Minimum difference required for statistical significance | | | | | | | | | 1.04 |

these sugars catabolized during work, the excess R.Q. is corrected for the increase due to the sugar alone, that is, by subtracting from the excess R.Q., the difference between the pre- and post-sugar resting R.Q.'s. When the excess R.Q. after sucrose is corrected in this way, the result, 0.85, is even lower than the pre-sugar R.Q. With glucose, however, this calculation gives a corrected excess R.Q. of 0.92, which is clearly higher than the control pre-sugar value. This indicates

TABLE 2

*The R.Q. and per cent net efficiency before and after glucose.*

| SUBJECT R.K. THROUGHOUT | | BEFORE GLUCOSE | | | | AFTER GLUCOSE | | | |
|---|---|---|---|---|---|---|---|---|---|
| Date | Syrup | Rest | R.Q. Work and recovery | Excess | Per cent net efficiency | Rest | R.Q. Work and recovery | Excess | Per cent net efficiency |
| 2/28 | Sweetose | .83 | .97 | 1.06 | 20.28 | | | | |
| 3/ 1 | 50 gm. | .90 | .87 | .86 | 20.63 | .88 | .95 | 1.00 | 21.75 |
| 3/ 4 | 50 gm. | .97 | .90 | .86 | 21.33 | .96 | .92 | .90 | 21.27 |
| 3/ 5 | 50 gm. | .86 | .83 | .82 | 20.91 | .86 | .92 | .97 | 21.47 |
| 3/ 6 | 50 gm. | .79 | .83 | .85 | 20.35 | .82 | .92 | .98 | 21.23 |
| 4/26 | 50 gm. | .83 | .84 | .88 | 20.62 | .88 | .90 | .91 | 20.15 |
| 4/29 | 50 gm. | .98 | .88 | .77 | 21.11 | 1.01 | .97 | .88 | 20.36 |
| 5/ 1 | 102 gm. | .92 | .88 | .87 | 20.47 | .94 | .93 | .92 | 21.14 |
| 5/ 2 | 102 gm. | .89 | .88 | .88 | 20.40 | .88 | .89 | .90 | 21.01 |
| 5/ 3 | 102 gm. | .96 | .87 | .85 | 21.43 | .87 | .91 | .93 | 20.54 |
| 5/ 8 | Corn syrup | .86 | .87 | .86 | 21.29 | .91 | .90 | .89 | 22.56 |
| 5/ 9 | 50 gm. | .94 | .88 | .84 | 21.50 | .86 | .91 | .94 | 20.86 |
| 5/12 | 50 gm. | .86 | .88 | .90 | 21.93 | .93 | .92 | .91 | 21.69 |
| 5/15 | 50 gm. | .87 | .86 | .86 | 21.00 | .92 | .92 | .92 | 21.58 |
| Average | | .89 | .87 | .87 | 21.09 | .90 | .92 | .93 | 21.20 |
| Net increase ("after" minus "before sugar") | | | | | | .01 | .05 | .06 | .11 |
| Difference required for significance | | | | | | | | | .73 |

that more glucose than sucrose is metabolized during work, a fact which is not apparent from a comparison of the uncorrected excess R.Q.'s, in which sucrose is higher.

### Muscular efficiency

The percentage net efficiencies, obtained by dividing the heat of the mechanical work done by the excess energy caused by this work, are included in tables 1 and 2. Although there is an increase after both sugars, the increase after sucrose narrowly misses significance, whereas the increase after glucose is clearly not significant.

### DISCUSSION

Krogh and Lindhard ('20) have suggested that an inter-conversion of fats and carbohydrates occurs, depending on

their relatively available quantities in the body. When the supply of carbohydrate is in excess of that of fat, fat is formed from carbohydrates. This occurs at R.Q.'s above 0.90. The reverse occurs when the quotient is below 0.80. During work, this anabolic process continues unaltered or may possibly be reduced; at least, it is not increased in proportion to the increased catabolism. Its relative effect on the total R.Q. of work is diminished, causing the R.Q. to rise when it is low during the pre-work rest period, and fall when it is high at rest. The R.Q. arrangement shown in figures 1 and 2 would seem to be a good illustration of this hypothesis. As most of the baseline R.Q.'s cluster around 0.88 (fig. 1), it might be said that the normal equilibrium in this particular nutritive state occurs at this value, when, according to the Zuntz-Schumburg tables, the proportion of carbohydrate to fat burned would be 59 to 41. When either fat or carbohydrate is in excess, the equilibrium is destroyed and in an attempt to restore the balance, the rest R.Q. will be raised or lowered according to the direction of the fat ⇆ carbohydrate conversion. During work, when the catabolic phase is stressed, the work R.Q. value should approach the equilibrium value of 0.88. Such changes were observed. The work and recovery values as shown here all lie very close to 0.88, seldom going over 0.91 or below 0.85. The excess R.Q.'s, however, should vary in exactly the reverse direction from the rest R.Q.'s. This was also observed and can be seen from the descending trend from left to right in figure 1. The excess R.Q.'s corresponding to rest R.Q.'s of 0.87–0.89 are numerically identical. But from the indirect method of calculating this value $\frac{\text{Work } CO_2 - \text{Rest } CO_2}{\text{Work } O_2 - \text{Rest } O_2}$ in cases where there is a large change, either increase or decrease, from the rest R.Q. to a value of 0.88 in the work and recovery period, there will be, of necessity, an even larger change in the same direction in the excess R.Q.

Thirty minutes after the ingestion of sugar (fig. 2) a nutritional state exists in which all of the relations between the various R.Q.'s found in the baseline experiments are essentially repeated. However, the equilibrium is now established

at an R.Q. of about 0.92, at which 73% carbohydrate to 26% fat is burned.

The results obtained on the effect of various sugars on the R.Q. are in agreement with those of the workers cited above. There is a greater rise in R.Q. after sucrose, due to its fructose component, which according to their interpretation indicates conversion of this sugar to fat. It is also shown that more glucose than sucrose is metabolized during work.

Carpenter and Fox and others, and Bachmann, Haldi and associates, were unable to find any change in muscular efficiency after ingestion of various sugars, regardless of the quantities taken or the time intervals after ingestion. The latter workers repeated Haggard and Greenberg's experiments under nearly identical circumstances, but were unable to confirm the striking and apparently consistent increases reported in that investigation. The data in this paper add confirmation to the reports of Carpenter, and Bachmann and Haldi, since no statistically significant increases in the mechanical efficiency were observed following the ingestion of sugars.

SUMMARY

1. The rest R.Q.'s 6–8 hours after the last meal with no control of the diet vary over a wide range, from 0.69 to 1.00. When the rest R.Q.'s are arranged numerically, the work and recovery, and excess R.Q. values increase if their corresponding rest R.Q. is below 0.88 for pre-sugar experiments or below 0.92 for post-sugar experiments, and decrease if their rest R.Q. is above these figures. These data are interpreted by the hypothesis that when fat is in excess it is converted to carbohydrate and stored, and when carbohydrate is preponderant, the reverse occurs.

2. Sucrose, probably because of the conversion of its fructose component to fat, raises the rest R.Q. higher than do the glucose corn syrups.

3. Following the ingestion of glucose and of sucrose, the excess R.Q.'s, when corrected for the increase due to sugar

alone, show that more glucose than sucrose is metabolized during work.

4. No significant changes in muscular efficiency occur after the ingestion of either of the sugars.

### LITERATURE CITED

BACHMANN, G., J. HALDI, W. WYNN AND C. ENSOR  1937  The respiratory quotient and carbohydrate metabolism following the ingestion of glucose and of fructose as affected by exercise taken immediately and thirty minutes after ingestion. Am. J. Physiol., vol. 120, p. 579.

CARPENTER, T. M., AND E. L. FOX  1931 a  The effect of muscular work upon the respiratory exchange of man after the ingestion of glucose and fructose. I. The respiratory quotient and the mechanics of breathing. Arbeitsphysiologie, vol. 4, p. 532.

———————  1931 b  II. Heat production, efficiency, oxygen debt, excess respiratory quotient and metabolism of carbohydrates. Ibid., p. 570.

CARPENTER, T. M., AND R. C. LEE  1938  The effect of muscular work on the metabolism of man after the ingestion of sucrose and galactose. Arbeitsphysiologie, vol. 10, p. 172.

HAGGARD, H. W., AND L. A. GREENBERG  1935  Diet and Physical Efficiency. The Influence of Frequency of Meals upon Physical Efficiency and Industrial Productivity. Yale University Press, New Haven, Conn.

HALDI, J., AND G. BACHMANN  1937  The effect of exercise on metabolism following the ingestion of water, glucose and fructose as shown by the course of the respiratory quotient. J. Nutrition, vol. 14, p. 287.

HALDI, J., G. BACHMANN AND C. ENSOR  1938  Muscular efficiency in relation to the taking of food and to the height of the respiratory quotient immediately before exercise. Am. J. Physiol., vol. 121, p. 123.

KROGH, A., AND J. LINDHARD  1920  The relative value of fat and carbohydrate as sources of muscular energy. Biochem. J., vol. 14, p. 290.

# THE THIAMINE REQUIREMENT OF THE ALBINO RAT AS INFLUENCED BY THE SUBSTITUTION OF PROTEIN FOR CARBOHYDRATE IN THE DIET [1]

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The first indication that the thiamine requirement might depend on the nature of the diet came in 1914 when Funk, and Braddon and Cooper discovered that diets high in carbohydrate hastened the outbreak of beriberi. More recently the problem has received the attention of Roche ('31), Peters ('36), Banerji and Harris ('39), and Arnold and Elvehjem ('39). Numerous workers, beginning with Platt and Lu ('35), have used the increases in both blood and urinary pyruvate and bisulphite-binding substances as indices of deranged carbohydrate metabolism in thiamine deficiency. The most recent studies have been those of Shils, Day and McCollum ('41), Harper and Deuel ('41), and Harper ('42) on various factors influencing the urinary excretion of these substances.

The relationship between thiamine and the protein of the diet has received considerable attention, and yet no one, except Funk and his colleagues (Funk, Collazo and Kaczmarek, '25), has suggested a role for protein analogous to the "sparing" role described by Evans and Lepkovsky ('28) for fat. Although Sherman and Gloy ('27), Hogan and Pilcher ('33) and Guerrant and Dutcher ('34) all reported that variations in the protein content of the diet had no effect on the vitamin $B_1$

requirement, Evans, Lepkovsky and Murphy ('34) found that for fat to exert its optimum sparing action, the protein content of the diet had to be high. More recently it has been demonstrated that the addition of vitamin $B_1$ to the vitamin B-complex-free diet of rats favors the free-selection of carbohydrate, and to a lesser but almost equally significant degree, the free-selection of protein (Richter and Hawkes, '41). It is probable, therefore, that thiamine participates in the metabolism of the proteins to the extent of the glycogenic amino acids which they contain.

The present investigation is concerned with the role of carbohydrate and protein in relation to the thiamine requirement of the adult, male albino rat. It is intended that the feeding of a high protein diet (64% casein) to rats which had previously received a high carbohydrate diet (64% sucrose) should reveal a decrease in the thiamine requirement. The change in the requirement should parallel the decrease in the glycogenic constituents of the diet.

### EXPERIMENTAL

The adult, male albino rats used in this experiment (Wistar Institute strain) were taken at random from among a large number of stock colony weanlings and maintained on commercial dog food [2] until approximately 9 months of age. They were then separated and placed in cylindrical wire-mesh metabolism cages in preparation for the experiment. The final distribution was not wholly a random one in that the six smallest animals were selected to make up the deficient and normal control groups.

The 24-hour urine collections were started at exactly 9 A.M., at which time the metabolism cages were placed on an 8-inch funnel into which a 7-inch circular screen of a mesh sufficiently small to catch the fecal pellets had been placed. The receiving vessel consisted of a 250 cc. Erlenmeyer flask. The trichloracetic acid (15 cc. of a 10% solution) used as a preservative was poured down the sides of the funnel so as to

[2] Purina dog chow.

coat the inner wall with acid. The small amount of urine that was caught in the meshes of the fecal-retaining screen dried there without the benefit of a preservative. The funnel and the screen were washed with approximately 100 cc. of distilled water at the end of the collection period and the combined urine, preservative, and washings were filtered superficially through cloth or coarse filter paper to remove the hair and spilled feed and then made up to a volume of 250 cc.

TABLE 1

*Composition of diets used.*

| INGREDIENTS | HIGH CARBO-HYDRATE [1] | HIGH PROTEIN |
|---|---|---|
| Basal mixture, in % : | | |
| Sucrose [2] ........................ . | 64.0 | 19.6 |
| Casein [3] ........................ | 20.0 | 64.0 |
| Hydrogenated cottonseed oil [4] ...... | 9.89 | 9.88 |
| Cellulose flour ........... ........ | 2.85 | 3.04 |
| Salts [5] ........................ | 3.00 | 3.20 |
| Supplements (per 10 kg. of basal mixture) : | | |
| Viosterol in oil | | |
| (10,000 I.U. per gm. oil) (gm.)... | 3.333 | 3.589 |
| Carotene in oil | | |
| (3.69 mg. per gm. oil) (gm.)..... | 8.000 | 8.615 |
| Riboflavin (mg.) ................ | 50.0 | 53.9 |
| Pyridoxine (mg.) ................ | 50.0 | 53.9 |
| Ca pantothenate (mg.) .. ......... | 24.0 | 25.9 |
| Lecithin (gm.) .................. | 15.00 | 16.14 |

[1] Gross energy value 4.7 calories per gram.
[2] Moisture content of: sucrose, 0.02%; casein, 4.09%.
[3] Labco, vitamin-free, from Borden Company.
[4] Crisco (10% diminished by oil in supplements).
[5] Hubbell, Mendel and Wakeman ('38).

The urinary pyruvate was determined by the colorimetric method of Lu ('39) with the slight modification that a compound Corning filter (nos. 502, 3.95 mm.; 430, 4.00 mm.; 315, 3.00 mm.) was used instead of the Wratten no. 62. One cubic centimeter of the collected urine that had been made up to a volume of 250 cc. was used for each duplicate determination. The composition of the diets is given in table 1.

Preliminary studies made with the high carbohydrate diet (table 1) revealed that:

(1) A 10% solution of trichloracetic acid, when used as a preservative, prevented the loss of a small amount of pyruvate during the 24-hour collection period. The freshly excreted urines of two rats were so divided that duplicate determinations for pyruvic acid could be made after the urines had been exposed to the atmosphere for 24 hours with and without trichloracetic acid. In both instances the untreated samples averaged 3% less pyruvic acid than did the treated samples.

(2) The normal excretion of two rats receiving 16 gm. of the diet daily was unchanged at the end of 4 weeks. The rats were apparently able to utilize the synthetic diet in a normal manner and the diet contained no unknown substances which might have surreptitiously influenced the pyruvate excretion.

(3) The thiamine requirement of adult, male rats (347 to 374 gm.) receiving 16 gm. of the diet was somewhat above 20 µg. of thiamine per day. Nine rats were divided into three groups of three rats each and given 10, 15 and 20 µg. of thiamine daily. All animals showed increased pyruvate excretions.

Eighteen mature, male rats (maintained at 26–30°C.) were used for the main experiment, of which three served as deficient controls (nos. 1–3, weighing 321 to 325 gm.), and three as normal controls (nos. 4–6; initial weight 308 to 339 gm.; final weight 375 to 396 gm.). Normal urinary pyruvate values were first determined for all rats following a week's feeding of the high carbohydrate, thiamine-supplemented diet (14 gm. containing 5 µg. of thiamine per gram of diet). The deficient controls were then transferred to a high carbohydrate diet wholly deficient in thiamine, and rats 7 to 18 (initial weight 332 to 414 gm.; final weight 384–437 gm.) were divided into four groups of three rats each. Following the 2-day depletion period during which they were given the deficient diet, the four groups were then supplemented with 18, 20, 22 and 24 µg. of thiamine daily. It soon became apparent that 24 µg. was not sufficient to meet the needs of the three animals on the highest

plane of intake and the intake of all groups was increased 8 µg. per day to 26, 28, 30 and 32 µg., respectively. In approximately 2 weeks it was clear that individual differences were greater than was first supposed, and the group plan was abandoned. From that time on each animal was treated individually.

The results obtained with the high carbohydrate diet are presented in table 2. The individual thiamine requirements in the last column are all estimated to be within at least 2 µg. of the true value. In only two cases (rats 10 and 13) did the final pyruvate values decrease to what the initial values had been. The final requirement for each of the remaining animals was estimated on the basis of former responses to the increased administration of thiamine, as well as on the basis of the performance of the group as a whole. The factors of time and the amount of available diet prevented the extension of the experiment for another 2 or 4 weeks.

The daily thiamine requirement values for rats 12 and 18 were not recorded (rat 18 being destroyed) because they refused food during the last week and during the final collection period. Not only has it been shown that the quantity of food metabolized influences the excretion of pyruvic acid (Shils, Day and McCollum, '41), but since the vitamin supplement was weighed separately and added to the diet each day, for which purpose casein served as a vehicle (1 mg. of casein contained 1 µg. of thiamine), there was no way of determining whether the supplement had been consumed.

The high protein diet on analysis was found to contain 5.485 calories per gram. Since the plan of the experiments called for isocaloric or near-isocaloric feeding, the high protein diet was first administered at the 12-gm. level of intake and not at the 13-gm. level as had been planned before the energy analysis was made. The immediate and numerous food refusals, which prompted a lowering of the food intake in order to stimulate the appetite, as well as the small but significant gains made when the animals were receiving the high carbohydrate diet in an isocaloric amount, suggested one conclusion:

## TABLE 2
### High carbohydrate diet:
### The relation of pyruvate excretion to the intake of thiamine.

| Rat No. | PERIOD 1 B₁/day 7 days | P.A.[1] in 24 hrs. | PERIOD 2 B₁/day 2 days | B₁/day 5 days | P.A. in 24 hrs. | PERIOD 3 B₁/day 7 days | P.A. in 24 hrs. | PERIOD 4 B₁/day 4 days | B₁/day 3 days | P.A. in 24 hrs. |
|---|---|---|---|---|---|---|---|---|---|---|
| | $\mu g.$ | $mg.$ | $\mu g.$ | $\mu g.$ | $mg.$ | $\mu g.$ | $mg$ | $\mu g.$ | $\mu g.$ | $mg.$ |
| 1 | 70 | 1.8 | 0 | 0 | 2.7[2] | 0[3] | 6.4[2] | 0[3] | 0[3] | 7.9[2] |
| 2 | 70 | 1.8 | 0 | 0 | 2.1 | 0 | 6.7[2] | 0[3] | 0[3] | 4.9[3] |
| 3 | 70 | 2.0 | 0 | 0 | 2.9 | 0 | 5.5 | 0[3] | 0[3] | 7.6[2] |
| 4 | 70 | 1.7 | 70 | 70 | 1.9 | 70 | 1.9 | 70 | 70 | 2.0 |
| 5 | 70 | 2.3 | 70 | 70 | 2.4 | 70 | 2.6 | 70 | 70 | 2.7 |
| 6 | 70 | 2.4 | 70 | 70 | 2.6 | 70 | 3.1 | 70 | 70 | 2.9 |
| 7 | 70 | 2.5 | 0 | 18 | 3.1 | 18 | 3.7 | 18 | 26 | 3.3 |
| 8 | 70 | 2.7 | 0 | 18 | 3.2 | 18 | 5.0 | 18 | 26 | 4.5 |
| 9 | 70 | 2.6 | 0 | 18 | 3.3 | 18 | 4.4 | 18 | 26 | 4.4 |
| 10 | 70 | 1.8 | 0 | 20 | 2.8 | 20 | 3.2 | 20 | 28 | 3.2 |
| 11 | 70 | 2.1 | 0 | 20 | 2.8 | 20 | 3.8 | 20 | 28 | 3.7 |
| 12 | 70 | 2.0 | 0 | 20 | 2.4 | 20 | 3.2 | 20 | 28 | 2.9 |
| 13 | 70 | 2.5 | 0 | 22 | 3.5 | 22 | 3.4 | 22 | 30 | 3.8 |
| 14 | 70 | 3.4 | 0 | 22 | 3.9 | 22 | 4.6 | 22 | 30 | 5.2 |
| 15 | 70 | 1.6[2] | 0 | 22 | 3.5 | 22 | 3.8 | 22 | 30 | 3.9 |
| 16 | 70 | 2.1 | 0 | 24 | 2.8 | 24 | 3.2 | 24 | 32 | 3.1 |
| 17 | 70 | 2.7 | 0 | 24 | 2.8 | 24 | 4.0 | 24 | 32 | 4.3 |
| 18 | 70 | 3.4 | 0 | 24 | 4.1 | 24 | 5.3 | 24 | 32 | 6.6 |

| Rat No. | PERIOD 5 B₁/day 7 days | P.A. in 24 hrs. | PERIOD 6 B₁/day 7 days | P.A. in 24 hrs. | PERIOD 7 B₁/day 7 days | P.A. in 24 hrs. | PERIOD 8 B₁/day 7 days | P.A. in 24 hrs. | DAILY B₁ REQUIREMENT |
|---|---|---|---|---|---|---|---|---|---|
| | $\mu g.$ | $mg.$ | $\mu g.$ | $mg$ | $\mu g.$ | $mg$ | $\mu g.$ | $mg.$ | $\mu g.$ |
| 4 | 70 | 1.9 | 70 | 2.1 | 70[3] | 1.8 | 70[3] | 2.1 | |
| 5 | 70 | 2.3 | 70 | 2.5 | 70 | 2.2 | 70 | 2.4 | |
| 6 | 70 | 2.9 | 70 | 2.6 | 70 | 2.4 | 70 | 2.8 | |
| 7 | 26 | 3.0 | 26 | 3.7 | 30 | 3.4 | 30 | 2.8 | 30 |
| 8 | 26 | 4.6 | 26 | 4.9 | 32 | 4.2 | 32 | 3.9 | 34 |
| 9 | 26 | 4.4 | 26 | 4.8 | 32 | 3.6 | 32 | 4.3 | 36 |
| 10 | 28 | 3.2 | 28 | 3.7 | 32 | 3.2 | 32 | 2.5 | 32 |
| 11 | 28 | 4.7 | 28 | 3.4 | 30 | 3.4 | 30 | 3.1 | 32 |
| 12 | 28 | 2.9 | 28 | 3.1 | 32 | 2.7 | 32[2] | (2.1)[2] | .. |
| 13 | 30 | 2.8 | 30 | 2.8 | 28 | 2.6 | 28 | 2.7 | 28 |
| 14 | 30 | 5.0 | 30 | 4.8 | 32 | 4.3 | 32 | 4.1 | 34 |
| 15 | 30 | 3.8 | 30 | 3.6 | 30 | 3.5 | 30 | 3.0 | 32 |
| 16 | 32 | 3.2 | 32 | 3.6 | 34 | 2.7 | 34 | 2.8 | 36 |
| 17 | 32 | 4.3 | 32 | 3.8 | 32 | 3.2 | 32 | 3.5 | 34 |
| 18 | 32 | 5.6 | 32[2] | 4.6[2] | 30[3] | 3.8[2] | 30[3] | (4.7)[2] | .. |

Average = 33
(Coefficient of variation = 8%)

[1] Pyruvic acid (P.A.) determined on urine of last 24 hours in each period.
[2] Food refused during urine collection.
[3] Food refused at least once during period indicated; but not during urine collection, unless indicated.

that the 12-gm. level of intake was too high. Subsequently, the diet was fed at the 11-gm. level until the experiment ended. Thus, the animals received 60.3 calories per day when on the high protein diet as compared to 65.8 calories per day when on the high carbohydrate diet.

The daily thiamine supplements that were initially administered to the assay animals on the high protein diet were estimated with the aid of both the previous requirements on the high carbohydrate diet and the ratio of glycogenic substances contained in the two diets. The casein was held to be approximately 50% glycogenic on the basis of Schmidt's ('38) list of glycogenic amino acids, and Hawk and Bergeim's ('37) analysis of casein as adapted from Mitchell and Hamilton. The hydrogenated cottonseed oil [3] was considered to be at least 10% glycogenic by virtue of the glycerol it contained. It was estimated that the 12 gm. of the high protein diet initially fed would yield 6.17 gm. of glycogenic substances as compared to 10.45 gm. contained in the 14 gm. of the high carbohydrate diet. This ratio approximates 0.60.

The results obtained with the high protein diet are presented in table 3. The daily thiamine requirement values which constitute the last column are not, as previously, "estimated to be within at least 2 µg. of the true value." In this instance they are the exact amounts of thiamine which prevented any significant rise in the urinary pyruvate excretion.

The 50% decrease in the amount of thiamine administered to a number of the rats during the first week attended the lowering of the food intake for a few days in order to stimulate the appetite. Rat 5 was destroyed and replaced by another normal control, rat 5 A, because it refused food and lost weight. The fact that its urine contained blood suggested some renal injury. A gross examination, however, revealed no obvious defect. The requirement values for rats 11 and 12 are missing because they also refused food; as a consequence of their loss of appetite, they were killed. Their pyruvate

[3] Crisco.

## TABLE 3

### *High protein diet:*

### *The relation of pyruvate excretion to the intake of thiamine.*

| PERIOD | 1 | | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|---|
| Rat No. | $B_1$/day | | P.A.[1] in 24 hrs. | $B_1$/day | P.A. in 24 hrs. | $B_1$/day | P.A. in 24 hrs. | $B_1$/day | P.A. in 24 hrs. |
| | 5 days | 2 days | | 6 days | | 5 days | | 6 days | |
| | *μg.* | *μg.* | *mg.* | *μg.* | *mg.* | *μg.* | *mg.* | *μg.* | *mg.* |
| 4 | 60[3] | 60[3] | (3.1)[2] | 55[4] | 3.8 | 55 | 3.7 | 55 | 3.5 |
| 5 | 60[3] | 60[3] | (3.0)[2] | 55[4] | 3.8 | 55[3] | 3.6 | 55[3] | (2.9)[2] |
| 6 | 60 | 60 | 3.5 | 55 | 3.4 | 55 | 3.5 | 55 | 3.4 |
| 7 | 18[3] | 9[4] | (3.2) | 18 | 4.0 | 18 | 4.3 | 18 | 4.6 |
| 8 | 20[3] | 10[4] | (3.5) | 20 | 3.7 | 20 | 4.1 | 20[3] | 4.0 |
| 9 | 22 | 22 | 3.9 | 22 | 3.5 | 22 | 4.0 | 22 | 4.0 |
| 10 | 19[3] | 10[4] | 3.5 | 19 | 3.5 | 19 | 4.1 | 19 | 3.8 |
| 11 | 19[3] | 10[4] | 3.4 | 19 | 3.2 | 19 | 3.5 | 19[3] | (2.9)[2] |
| 12 | 19[3] | 10[4] | 3.3 | 19 | 3.5 | 19 | 3.8 | 19 | 3.8 |
| 13 | 17 | 17 | 3.8 | 17 | 3.8 | 17 | 3.7 | 17 | 3.7 |
| 14 | 20[3] | 10[4] | 4.3 | 20 | 4.2 | 20 | 4.0 | 20 | 4.0 |
| 15 | 19 | 19 | 3.7 | 19 | 3.7 | 19 | 3.8 | 19 | 4.3 |
| 16 | 22 | 22 | 3.4 | 22 | 3.5 | 22 | 3.5 | 22 | 3.6 |
| 17 | 20[3] | 10[4] | (3.2)[2] | 20[3] | 3.5[2] | 20 | 3.5 | 20 | 3.8 |

| PERIOD | 5 | | 6 | | 7 | | DAILY $B_1$ REQUIREMENT |
|---|---|---|---|---|---|---|---|
| Rat No. | $B_1$/day | P.A. in 24 hrs. | $B_1$/day | P.A. in 24 hrs. | $B_1$/day | P.A. in 24 hrs. | |
| | 6 days | | 6 days | | 7 days | | |
| | *μg.* | *mg.* | *μg* | *mg.* | *μg.* | *mg.* | *μg.* |
| 4 | 55 | 3.4 | 55[3] | 3.5 | 55[3] | 3.5[2] | |
| 5 A[5] | 55[4] | 3.2 | 55 | 3.2 | 55 | 3.7 | |
| 6 | 55 | 3.4 | 55 | 3.5 | 55 | 3.6 | |
| 7 | 20 | 4.1 | 20 | 5.0 | 22 | 4.6 | 22 |
| 8 | 18 | 4.2 | 18 | 4.8 | 20 | 6.1 | 22 ? |
| 9 | 20 | 4.3 | 20 | 4.0 | 18 | 4.6 | 20 |
| 10 | 17 | 4.4 | 17 | 4.6 | 19 | 4.8 | 19 |
| 11 | Killed | | | | | | |
| 12 | 17 | 3.7 | 15 | 3.7[2] | 15[3] | (4.1)[3] | .. |
| 13 | 15 | 4.0 | 15 | 4.5 | 15 | 4.4 | 17 |
| 14 | 18 | 4.2 | 18 | 4.4 | 18 | 4.1 | 18 |
| 15 | 19 | 4.4 | 21 | 4.4 | 21 | 4.3 | 21 |
| 16 | 20 | 3.6 | 20 | 3.5 | 18 | 3.5 | 18 |
| 17 | 18 | 4.1 | 18 | 4.1 | 20 | 3.7 | 20 |

Average = 20
(Coefficient of variation = 9%)

[1] Pyruvic acid (P.A.) determined on urine of last 24 hours in each period.

[2] Food refused during urine collection.

[3] Food refused at least once during period indicated; but not during urine collection, unless indicated.

[4] Food intake lowered to stimulate appetite.

[5] Rat 5 A, a control, substituted for rat 5 which refused food and lost weight.

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values remained within normal range at all times so that a thiamine insufficiency was not the direct cause of their anorexia.

<center>DISCUSSION</center>

The deficient controls (rats 1–3) on the high carbohydrate diet reacted in a manner characteristic of a developing thiamine deficiency. They showed a fourfold increase in the excretion of urinary pyruvic acid in 3 weeks' time, although the final value for rat 2 was somehow unaccountably decreased. These regular increases offered some assurance that the deficient diet was, in fact, wholly devoid of, or at least very low in, thiamine.

The daily pyruvate excretions of the normal controls (high carbohydrate diet) were satisfactorily constant in spite of the fact that the collection represented only one 24-hour period out of 7 days. The excretion of rat 4 varied from 1.7 to 2.1 mg., that of rat 5 from 2.2 to 2.7 mg., and that of rat 6 from 2.4 to 3.1 mg. Some of the variation was due no doubt to the changes in body weight which approximately paralleled the pyruvate excretions: average initial weight, 327 gm., average initial pyruvate, 2.1 mg., average final weight, 385 gm., average final pyruvate, 2.4 mg.

The average daily thiamine requirement of 33 µg. (coef. var. $= 8\%$) as given in table 2 is considerably above the value of 14 µg. which is obtained on the basis of Arnold and Elvehjem's ('39) suggestion that "the requirement for thiamine by most species of animals may be simply stated as approximately 1 microgram of the vitamin per gram of dietary solids." It is also higher than the value obtained by Mills ('41) who produced normal growth at 65°F. but not at 91°F. when he fed 0.6 to 0.8 µg. of thiamine per gram of diet as a supplement to the same diet that was used by Arnold and Elvehjem. Previously, Van Veen ('32) had found that young rats weighing less than 100 gm. required from 4 to 8 µg. of thiamine daily.

The disagreement is not wholly unexpected since, in the above investigations the rate of growth was used as a measure

of thiamine deficiency, whereas it is suggested by this investigation that the increase in urinary pyruvate serves as a much more delicate index. The animal organism apparently responds with an increase in pyruvate excretion before it shows any symptoms of anorexia. This is borne out by a consideration of the deficient controls. Rat 1 showed an increase in urinary pyruvate from a normal value of 1.8 µg. to a deficient value of 2.7 µg. in 1 week's time and refused food only on the seventh day. Rat 2 showed an increase from 1.8 to 6.7 µg. in 2 weeks' time and did not refuse food before the fourteenth day. Rat 3 almost tripled its pyruvate excretion in 14 days without exhibiting any signs of anorexia.

The pyruvate excretion of the normal controls was more constant on the high protein diet than it had been on the high carbohydrate diet. The excretion of rat 4 varied between 3.4 and 3.8 mg., and that of rat 6 between 3.4 and 3.6 mg. The first excretion value for rat 4 (3.1 mg.) is not included because the food intake was lowered during that collection period in order to stimulate the appetite.

The initial, basal pyruvate excretion of most of the animals was higher on the high protein diet than on the high carbohydrate diet. All of the normal controls and many of the assay animals responded with immediate increases when changed to the high protein diet. The remaining animals showed either a decrease or no change, depending on whether their final value when receiving the high carbohydrate diet had been high or only moderately high.

The average daily thiamine requirement of 20 µg. (coef. var. = 9%) is 39% lower than the 33 µg. (coef. var. = 8%) needed when a high carbohydrate diet is fed. On the strength of the data given above, that casein is approximately 42% glycogenic (50% corrected for nitrogen) and fat at least 10% glycogenic, the decrease was estimated at 50%. This change in the requirement is in agreement with the results of Funk, Collazo and Kaczmarek ('25). They found that pigeons were able to metabolize more protein than carbohydrate when given the same amount of vitamin B.

If, at this time, for purposes of approximation only, it is permissible to ignore the fat fractions of the two diets since they occur in but small and near-equal amounts, then it can be calculated, as shown below, that casein should not be more than 64% glycogenic. The equations:

$$8.96 \, x + 2.69 \, y = 33$$
$$2.16 \, x + 6.75 \, y = 20$$

yield values of 3.09 and 1.97 for x and y, respectively, where x and y are the micrograms of thiamine required per gram of sucrose and casein contained in the diets and their coefficients the grams of each of these substances that were fed each day (dry weight). The ratio $1.97:3.09 = 64:100$. Although the total thiamine requirement values include the thiamine excreted in the urine and feces, any deductions for these factors would serve only to decrease the 64:100 ratio, since the excretion on the high protein diet must be equal to or less than that on the high carbohydrate diet; hence, the limitation, "not more than 64% glycogenic."

Under these circumstances a comparison of the two values, of which one (not more than 64% glycogenic) was obtained from experimental results, and the other (42% glycogenic) was calculated on the basis of data given by Schmidt ('38) and Hawk and Bergeim ('37), suggests that the glycogenic constituents of the diet are of major importance when studying thiamine requirement values. More precisely, it might be inferred that thiamine participates in the oxidation of the proteins to the extent of the glycogenic amino acids which they contain.

## SUMMARY

The average daily thiamine requirement of ten, adult, male albino rats of the Wistar Institute strain weighing nearly 400 gm. and maintained in approximate energy equilibrium at 26°–30°C. on a diet containing 64% sucrose, 20% casein, and 10% hydrogenated cottonseed oil (plus cellulose flour, salts, and pure vitamin supplements) has been found to be 33 µg.

(coef. var. $= 8\%$). On a diet high in protein (64% casein, 19.6% sucrose, 10% hydrogenated cottonseed oil; and representing a 9% decrease in the energy intake), nine of the ten rats had an average requirement of 20 µg. (coef. var. $= 9\%$).

A comparison of the two values demonstrates a 39% reduction in the daily requirement.

The increase in the urinary pyruvate excretion (determined by the method of Lu) which indicates a possible disturbance in intermediary carbohydrate metabolism has been used as the index of subacute thiamine deficiency.

LITERATURE CITED

ARNOLD, A., AND C. A. ELVEHJEM 1939 Influence of the composition of the diet on the thiamine requirement of dogs. Amer. J. Physiol., vol. 126, pp. 289–298.

BANERJI, G. G., AND L. J. HARRIS 1939 Methods for assaying the level of nutrition. A carbohydrate tolerance test for vitamin $B_1$. 1. Experiments with rats. Biochem. J., vol. 33, pp. 1346–1355.

BRADDON, W. L., AND E. A. COOPER 1914 The influence of metabolic factors in beri-beri. I. The effect of increasing the carbohydrate ration on the development of polyneuritis in birds fed on polished rice. J. Hygiene, vol. 14, pp. 331–353.

EVANS, H. M., AND S. LEPKOVSKY 1928 Sparing action of fat on the antineuritic vitamin. Science, vol. 68, p. 298.

EVANS, H. M., S. LEPKOVSKY AND E. A. MURPHY 1934 The sparing action of fat on vitamin B. VI. The influence of the levels of protein and vitamin G. J. Biol. Chem., vol. 107, pp. 429–437.

FUNK, C. 1914 Studien über Beriberi. XI. Die Rolle der Vitamine beim Kohlenhydrat-Stoffwechsel. Z. physiol. Chem., Bd. 89, S. 378–380.

FUNK, C., J. A. COLLAZO AND J. KACZMAREK 1925 Composition du regime et vitamine B. Compt. rend. soc. biol., t. 92, pp. 997–998.

GUERRANT, N. B., AND R. A. DUTCHER 1934 Some effects of the composition of the diet on the vitamin B and vitamin G requirement of the growing rat. J. Nutrition, vol. 8, pp. 397–420.

HARPER, H. A. 1942 Carbohydrate metabolism in thiamine deficiency. J. Biol. Chem., vol. 142, pp. 239–248.

HARPER, H. A., AND H. J. DEUEL, JR. 1941 The urinary pyruvate in thiamine deficiency. J. Biol. Chem., vol. 137, pp. 233–238.

HAWK, P. B., AND O. BERGEIM 1937 Practical Physiological Chemistry, 11th edition, p. 104. Blakiston, Philadelphia.

HOGAN, A. G., AND R. W. PILCHER 1933 Effects of variations in the amounts of vitamin B and protein in the ration. Missouri Agric. Exper. Sta. Res. Bull., 195.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1938 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, pp. 273–285.

LU, G. D. 1939 Studies on the metabolism of pyruvic acid in normal and $B_1$-deficient states. I. A rapid, specific and sensitive method for the estimation of blood pyruvate. Bioch. J., vol. 33, pp. 249–254.

MILLS, C. A. 1941 Environmental temperatures and thiamine requirements. Amer. J. Physiol., vol. 133, pp. 525–531.

PETERS, R. A. 1936 The biochemical lesion in vitamin $B_1$ deficiency. Application of modern biochemical analysis in its diagnosis. Lancet, vol. 1, p. 1161.

PLATT, B. S., AND G. D. LU 1935 Intermediate carbohydrate metabolism in vitamin $B_1$ deficiency in man. Chinese J. Physiol., Proc., vol. 9, p. 401.

RICHTER, C. P., AND C. D. HAWKES 1941 The dependence of the carbohydrate, fat and protein appetite of rats on the various components of the vitamin B complex. Amer. J. Physiol., vol. 131, pp. 639–649.

ROCHE, J. 1931 Sur l'accumulation dans le sang de corps ternaires au cours de l'avitaminose B. Bull. Soc. Chim. Biol., t. 13, pp. 186–196.

SCHMIDT, C. L. A., EDITOR 1938 The chemistry of the amino acids and proteins, p. 223. C. C. Thomas, Springfield, Illinois.

SHERMAN, H. C., AND O. H. M. GLOY 1927 Vitamin B determination and requirement with special reference to protein intake. J. Biol. Chem., vol. 74, pp. 117–122.

SHILS, M. E., H. G. DAY AND E. V. McCOLLUM 1941 The effect of thiamine deficiency in rats on the excretion of pyruvic acid and bisulphite-binding substances in the urine. J. Biol. Chem., vol. 139, pp. 145–161.

VAN VEEN, A. G. 1932 Das antineuritische Vitamin aus Reiskleie und die Polyneuritis der Versuchs-Tiere (8. Mitteilung über das antineuritische Vitamin aus Reiskleie). Mededeel. v. d. Dienst der Volksgezondheid in Ned.-Indië., Teil IV, S. 184–197.

# THE RESPIRATORY QUOTIENT OF THE LIVER

I. DETERMINATION OF THE LIVER R.Q. IN ANESTHETIZED DOGS

II. INFLUENCE OF CERTAIN DIETS ON THE R.Q.

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With one exception (Blalock and Mason, '36), previous determinations of the respiratory quotient of the liver have been made on the artificially perfused organ or on tissue slices in the Warburg apparatus. In the perfusion technique (Blixenkrone-Møller, '38), no care was taken either to approximate the normal blood flow through the organ or to maintain normal levels of blood gas tension in the perfusion fluid. The respiratory quotients so determined usually showed a progressive decrease during the course of an experiment. The results obtained from tissue slices (Gemmill and Holmes, '35; Stadie, Zapp and Lukens, '40) possibly may not be representative of the total metabolic processes which occur normally. Those results obtained from the livers of normal, unanesthetized dogs (Blalock and Mason) vary over such a wide range that little confidence can be placed in a single determination.

It was with the view to developing a technique by which consistent determinations of the respiratory quotient of the liver could be made under better physiological conditions than heretofore that this study was undertaken.

[1] In partial fulfillment of the requirements for the degree of Doctor of Philosophy.

## I. DETERMINATION OF THE LIVER R.Q. IN ANESTHETIZED DOGS

In order to estimate the R.Q. of the liver from a knowledge of the A-V gas differences and without knowing the total volume flow of blood, it is necessary to convert the double blood supply into a single one. Complete removal of one or the other source of inflowing blood is unsatisfactory since animals so treated show varying degrees of metabolic disturbances ranging from rapid death in the case of hepatic arterial ligation (Haberer, '06; Collens, Shelling and Byron, '27; Cameron and Mayes, '30) to less dramatic results in the case of the Eck fistula animal (Crandall and Roberts, '36; Smith and Whipple, '30). A single inlet may be attained, however, by anastomosing the common hepatic artery into the portal vein so that the liver receives both arterial and venous blood through one vessel.

This operation has been performed with some degree of success in the survival animal. Narath ('16) reported a few successful experiments in which the hepatic or renal artery was anastomosed into the portal vein. He concluded that such an anastomosis will serve to maintain the life of the animal and prevent the usual liver necrosis following complete exclusion of the normal hepatic arterial blood supply.

Rhoads and Chambers (unpublished observations) have attempted this operation in a number of dogs and succeeded in establishing chronically functional anastomoses in two animals which survived for more than a year, after which they were sacrificed. In all the other attempts, the dogs exhibited the usual pathological changes of the liver accompanying acute ligation of the hepatic artery, and were found to have completely thrombosed anastomoses. During the survival period in the two successful cases, no apparent clinical symptoms were manifested; blood sugar levels were normal and bromsulfalein tests showed no abnormality. It would appear from these results, that, if a successful anastomosis is attained, this operation will protect the animal against the harmful effects of hepatic artery ligation and will cause no gross changes in activity.

In view of the extreme technical difficulty of producing a chronically patent union between the hepatic artery and portal vein, it seemed advisable first to attempt the procedure in acute experiments, using a suitable anti-coagulant to maintain the anastomosis.

Dogs anesthetized with nembutal given intravenously were used. When required, the anesthesia was reinforced by intravenous barbital. Through a midline incision from xiphoid to umbilicus a clamp was placed on the common hepatic artery close to its origin from the celiac artery and a ligature tied about 3 cm. distal to this clamp; a metal cannula which pointed centrally was inserted between the clamp and ligature. The superior pancreatico-duodenal vein was ligated and a similar cannula, connected to the first by a short length of rubber tube, was inserted so that its opening projected well into the lumen of the portal vein. Chlorazol pink purified according to the method of Modell ('39) was injected intravenously, and the clamp on the hepatic artery released allowing arterial blood to pass into the portal vein. The hepatic artery proper was clamped just centrally to its first hepatic branch to prevent the very considerable collateral flow through the gastro-duodenal artery which otherwise would occur.

In most of the experiments, finely-ground cinnabar was injected intra-arterially at the conclusion of the determination to check the absence of collateral circulation. Fine collaterals usually could be seen extending throughout the gastrohepatic omentum and surrounding the sheath of the gall bladder. No cinnabar was ever found in the blood in the deep tissue of the liver, and it is felt that the slight degree of collateral circulation described is insufficient to cause any detectable error in the determination.

A 1½ inch length of 18-gauge hypodermic needle, attached to a rubber tube, was inserted into the portal vein so that the point could be pushed above the entrance of arterial blood. A single suture served to hold the needle in place against the movements of the surrounding viscera. The socket of the needle was attached to the other end of the tube and led out

through the abdominal incision so that the manipulation of attaching a syringe would not tear the needle out of the vein.

A second 1½ inch length of 18-gauge hypodermic needle was bent in a flame to an approximate right angle and a rubber tube attached to the blunt end. Insertion of this needle, which is a rather delicate procedure, was made by gently retracting the liver caudally with one hand until a good exposure of the hepatic vein was secured. With the aid of a long hemostat the needle was inserted through the ventral wall of the vein, about 1 cm. cephalic to its junction with the liver tissue. The rubber tube was led out through the incision, taking care that it remained between the midline of the diaphragm and the liver as that organ was allowed to fall back into place, attached to the socket of the needle and fastened to the skin in the region of the xiphoid. No suture is necessary to hold the needle in the vein as the wall of that vessel is sufficiently thick to prevent its slipping out. When both needles are correctly in place there should be a spontaneous flow from the socket in the case of the portal vein and little resistance should be met when drawing blood into a syringe in the case of the hepatic vein. Except when drawing samples, the rubber tube is clamped tightly near the socket in each case.

A cannula was inserted in the trachea, and the blood pressure was recorded by a Hürthle manometer connected to a cannula in the carotid artery.

Samples of blood were obtained as follows: 1½ cc. was drawn from the portal vein, immediately followed by a similar volume from the hepatic vein. Each sample was transferred anaerobically to a mercury bulb and kept under positive pressure in an ice-bath until analyzed. This procedure was repeated at intervals of about 1½ minutes until twelve samples from each vessel had been collected and combined in the storage bulbs. As an additional precaution against clotting, approximately ½ cc. of 30% potassium oxalate was added to each reservoir. During the drawing of the blood samples, the expired air was collected in a Tissot spirometer connected to the tracheal cannula by a set of rubber flap valves previ-

ously tested for leaks. Inspired air was room air. The total time taken to make one complete collection of blood and expired air usually amounted to between 15 and 20 minutes.

A second determination as a check was made whenever possible about 15 minutes following the first, and after the blood which had been withdrawn had been replaced by citrated blood from a donor animal. Due to the unusually great loss of blood from the abdominal wound in the presence of the anti-coagulant, the amount of blood injected usually was two or three times that represented by the samples.

This method of drawing the samples differs somewhat from that used by Himwich and Castle ('27) in the case of muscle. These workers drew a single large sample simultaneously from the artery and vein, but it is the author's opinion that a large number of very small samples over a relatively long time yields more consistent results than a single large sample over a short period. An analogy lies in the usual method of collecting expired air for determination of oxygen consumption. An additional advantage of using the small-samples technique lies in circumventing the error caused when the blood is either accumulated in or drained from the organ. Edmunds ('15), Bauer, Dale, Poulsson and Richards ('32) and others have presented evidence that the liver may act as a temporary blood reservoir. Since a requirement for successful determination of the R.Q. of a single organ from the A-V gas differences is that the total inflow and outflow of blood be equal during sampling, storage of blood in the organ during the drawing of the samples could cause a serious error in the result; but if the sampling is prolonged, such an error will be avoided since no organ can change its volume in one direction indefinitely.

The oxygen and carbon dioxide contents of the blood were determined in duplicate by the Van Slyke manometric method (Van Slyke and Neill, '24). Oxygen and carbon dioxide capacities were determined by equilibrating 10 cc. portions of the blood with 5% carbon dioxide and 95% oxygen at room temperature and analyzing in the Van Slyke apparatus. R.Q.'s

were calculated after correcting the gas content for possible change in hemoglobin concentration in passing through the liver, according to the method of Himwich and Castle. Expired air samples were collected from the spirometer in Bailey bottles and kept under positive pressure until analyzed in duplicate for oxygen and carbon dioxide in the Haldane-Henderson apparatus (Henderson, '18). The R.Q.'s were calculated in the usual way. In a few instances total acetone bodies were determined by Van Slyke's method (Van Slyke and Fitz, '17) and the total fatty acid content of the liver by the method of Leathes and Raper ('25).

The success of the method may be judged by reference to the first column of tables 1 and 2. Duplicate determinations of the R.Q.'s (represented by two sets of figures in one experiment) agreed within ±0.05 and in most cases within ±0.03, except in experiments 11 and 32; the maximum range found was 0.24 in experiment 11. The greatest standard deviation for a series of animals on a given diet was ±0.08. These values show a much greater degree of consistency than those obtained by Blalock and Mason on the livers of unanesthetized dogs, and by Himwich and Castle on resting muscle.

The cause of the exceptionally wide variation found in experiment 11 may have been due to the fact that the blood pressure had dropped to a level so low as to be indicative of a state of severe shock. In such a condition, carbohydrate may have been mobilized, and the high R.Q. simply reflects the participation of the liver tissue in the burning of this carbohydrate. The great variation in experiment 32 undoubtedly was due in part to the unusually low A-V gas differences, such low values serving to exaggerate the effect on the R.Q. of the analytical error of ±0.2 volumes per cent.

## II. INFLUENCE OF CERTAIN DIETS ON THE R.Q.

Previous to the experiment the animals were subjected to four different types of dietary regimes. The first type consisted of canned dog food given during at least a week previously; animals on this diet were in the post-absorptive state

(20 hours) before operation. The second type was starvation over a period of 2½ to 8 days. The third was a high fat diet in which approximately 60% of the daily caloric intake consisted of fat in the form of lard or corn oil, the remainder of the diet being canned dog food with added vitamins A, $B_1$, and D. The total caloric value of this ration approximated that of the first. In one case the diet consisted entirely of 100 gm. of corn oil daily, following 2 days of starvation. This was successful in only one instance, due to the weakness of the animal following a week or so of such treatment. The fourth type was identical to the high-fat diet with the exception of the addition of 2 gm. of choline chloride daily, in one case from the start of the regime and in another case for only the last 4 days. With the latter two diets, as with the first, the animals were in the post-absorptive state before operation.

In every case with the meat-fed, post-absorptive dog the value of the R.Q. fell within the physiological range. The average value of 0.80 is higher than most of those reported by Blixenkrone-Møller but it agrees fairly well with Blalock and Mason's average value of 0.74, and with Gemmill and Holmes' average value of 0.79 obtained on liver slices from rats fed normal diets.

There is no evidence in these results that, under normal conditions of diet, the majority of the metabolic processes of the liver of the dog are not carried to completion.

The average value of 0.59 obtained on the starvation diet is significantly lower than that of the post-absorptive, meat-fed animal. It may be of interest to point out that one of the determinations on this diet (0.61, experiment 15) was made while the hepatic artery proper was open. This experiment must be considered as technically in error due to the collateral pathway allowed through the open vessel, but it nevertheless seems to have had but slight influence on the determination.

Having no information other than the value of the R.Q., it is impossible to say anything positive concerning the cause of the low value in the case of this diet. R.Q.'s below 0.70 have frequently been cited as an indication of the trans-

TABLE 1

*R.Q.'s of dogs on a diet of canned food and starvation previous to operation.*

| DIET | R.Q. | | ARTERIAL-VENOUS DIFFERENCE | | MINUTE VOLUME |
|---|---|---|---|---|---|
| | Liver | Exp. air | $O_2$ | $CO_2$ | |
| | | | vol. % | vol. % | liter |
| I — Canned dog food | | | | | |
| Exp. 8 — 2/19/41 Body wt. 13.9 kg. | 0.76 | .... | 14.3 | 10.9 | .... |
| Exp. 11 — 3/20/41 Body wt. 9.8 kg. | 0.69 0.93 [1] | .... .... | 6.2 7.7 | 4.3 8.3 | .... .... |
| Exp. 12 — 3/27/41 Body wt. 9.1 kg. | 0.78 | .... | 9.1 | 7.1 | .... |
| Exp. 13 — 4/3/41 Body wt. 8.8 kg. | 0.85 | 0.80 | 5.3 | 4.5 | 4.17 |
| Exp. 20 — 6/9/41 Body wt. 14.9 kg. | 0.81 0.77 | 0.85 0.87 | 9.3 11.8 | 7.5 9.1 | 5.19 Lost |
| Exp. 22 — 6/16/41 Body wt. 9.3 kg. | 0.82 | 1.0 | 9.5 | 7.8 | 4.43 |
| Mean | 0.80 | | | | |
| Stand. deviation | ±0.07 | | | | |
| Mean of those in which exp. air was determined | 0.81 | 0.87 | | | |
| II — Starvation | | | | | |
| Exp. 15 — 4/17/41 Body wt. 12.5 kg. 2.5 days | 0.61 [2] 0.56 | 0.85 [2] 0.82 | 6.2 6.4 | 3.8 3.6 | 5.85 6.63 |
| Exp. 25 — 7/10/41 Wt. 7.1 kg. — loss of 1.4 kg. — 6.5 days | 0.66 0.57 | 0.86 0.77 | 7.3 6.7 | 4.8 3.8 | 3.36 3.69 |
| Exp. 26 — 7/24/41 Wt. 9.3 kg. — loss of 1.2 kg. — 8 days | 0.59 0.57 | 0.83 0.82 | 7.8 6.9 | 4.6 3.9 | 3.67 5.03 |
| Mean | 0.59 | | | | |
| Stand. deviation | ±0.04 | | | | |

[1] Animal in shock — blood pressure 59 mm. Hg.

[2] Not included in statistical analyses.

formation of fat into carbohydrate, a process which is said to have an R.Q. of less than 0.3 (Stadie, Zapp and Lukens, '41). In view of the evidence of Stadie, Zapp and Lukens ('40) and of Crandall, Ivy and Ehni ('40), however, it is more likely

TABLE 2

*R.Q.'s of dogs on a high fat and high fat + choline diet previous to operation.*

| DIET | R.Q. | | ARTERIAL-VENOUS DIFFERENCE | | BLOOD KETONES AS ACETONE | | LIVER FAT | MINUTE VOLUME |
|---|---|---|---|---|---|---|---|---|
| | Liver | Exp. air | $O_2$ | $CO_2$ | Portal | Hepatic | | |
| | | | vol.% | vol.% | mg.% | mg.% | % | liter |
| III — High fat | | | | | | | | |
| Exp. 24 [1] — 7/2/41 | | | | | | | | |
| 2 days fast, 5 days fat | 0.73 | 0.85 | 8.9 | 6.5 | .... | .... | .... | 3.44 |
| Wt. 6.7 kg. — loss 1.7 kg. | 0.78 | 0.86 | 8.5 | 6.6 | .... | .... | .... | 4.90 |
| Exp. 29 — 11/25/41 | 0.53 | .... | 3.0 | 1.6 | 14 on mixed bloods | | .... | .... |
| 22 days high fat | | | | | | | | |
| Wt. 11.0 kg. — loss 1.6 kg. | 0.49 | .... | 4.5 | 2.2 | | | | |
| Exp. 31 — 1/9/42 | 0.58 | 0.84 | 2.4 | 1.4 | 3 | 2 | 2.8 | .... |
| 39 days high fat | 0.68 | 0.90 | 3.1 | 2.1 | | | | |
| Mean | 0.57 | | | | | | | |
| Stand. deviation | ±0.08 | | | | | | | |
| IV — Choline | | | | | | | | |
| Exp. 30 — 12/2/41 | | | | | | | | |
| High fat + 2 gm. choline | 0.82 | .... | 9.9 | 8.1 | 1 | 2 | 9.7 | .... |
| Wt. 11.6 kg.—loss 1.0 kg. | 0.85 | .... | 12.8 | 10.9 | | | | |
| Exp. 32 — 1/16/42 | | | | | | | | |
| High fat 46 days 2 gm. | 0.63 | 0.82 | 2.4 | 1.5 | 13 | 1 | 3.5 | 2.90 |
| choline daily last 4 days. | 0.47 | 0.86 | 3.4 | 1.6 | | | | 3.13 |
| Wt. 11.0 kg. | | | | | | | | |

[1] Not included in statistical analyses.

that the R.Q. of 0.59 represents the production of ketone bodies from fat by the liver. Such a process has an R.Q. of 0.65 according to the theory of the successive B-oxidation of the fat molecule, and zero according to the theory of multiple alternate oxidation. Thus, if the liver were putting out ketone bodies,

the expected value of the R.Q. of the organ might well fall below 0.70, or the value for the complete oxidation of the fat molecule.

The average value of 0.57, obtained on animals on the high fat diet in which 60% of the caloric intake was in the form of lard or corn oil, is significantly lower than that of the meat-fed dog but is not significantly different from that on the starvation diet. In experiment 29, the high value of ketones found in the blood lends support to the possibility that this low R.Q. is representative of the formation of ketones from fat by the liver. Unfortunately the analysis for liver fat was lost in this instance, but it is worthwhile mentioning that the liver of this animal had the yellowish color typical of livers with high fat content. This was the only instance where such a condition was so pronounced as to be worthy of note. In experiment 31 the results of the blood ketone and liver fat analyses do not indicate that the animal had anything but a normal level of fat in the liver and scarcely any ketone production. Duplicate analyses of acetone and fat agreed very well in this case, but the methods used may have yielded different results in more experienced hands. Quite possibly the R.Q. may be a much more sensitive indicator of the general course of fat metabolism in the liver than measurements of the ketone body production or fat level.

In the single instance where the diet consisted of 2 days of starvation followed by 5 days of pure fat feeding, the R.Q.'s were above 0.70. In view of the preceding results the reason for this is a complete mystery. Two attempts were made to obtain additional information on this point, but for reasons previously given further trials were abandoned. This result should be checked in the future.

In the single experiment performed with a diet of high fat supplemented with 2 gm. of choline chloride daily, the R.Q. of the liver was 0.84. The blood ketone analysis indicated no abnormal production of ketones by the liver, yet the liver fat was at the abnormally high level of 9.7%. On the basis of this single experiment, it cannot be said that choline

given in conjunction with a high fat diet will prevent ketone production from fat by the liver. It is felt that these results are of sufficient interest to warrant further intensive investigation of this subject; it is surprising that the liver fat was so high in this instance. In the face of other evidence (Best, Ferguson and Hershey, '33) on the effect of choline on the accumulation of fat in the liver, these results in experiment 30 cannot be satisfactorily explained. Possibly it may be due to individual variation in the response of the animal to the substance.

In experiment 32, choline was given during the last 4 days of the high fat regime with the idea of investigating the possibility of the transformation of fat into carbohydrate as a partial explanation of the disappearance of fat from the livers of animals fed high fat diets followed by choline. If such a process occurred to any appreciable degree, the R.Q. of livers so treated should fall to a significantly lower level than that obtained on the animal fed a high-fat ration without the later addition of choline. As can be seen from the results, no indication of any such transformation was obtained. The average R.Q. was not appreciably different from those obtained on the high-fat, no-choline diets. This may have been due, however, to the failure of the liver to accumulate fat during the 42 days preceding the choline feeding.

The results of the blood ketones analysis in the case of experiment 32 are peculiar in that considerably more ketones were found in the blood entering the liver than in that leaving. This is completely unexplainable on the basis of any present evidence.

Another point of importance is the comparison of the R.Q.'s of the liver with those of the entire animal as obtained from the expired air. The mean of the expired air quotients of animals on the canned food diet was not significantly different from that of the liver quotients on the same diet (considering only those experiments where both liver and expired air quotients were obtained). It would appear from these results that the total metabolic oxidations occurring in the liver are

similar to those occurring in the entire animal in the case of meat-fed, post-absorptive dogs.

In the case of the starvation diet, however, there is a very real difference between the R.Q. of the liver and of the entire body. This difference can easily be explained on the basis that the end-products of whatever unusual metabolic process occurred in the liver to lower the R.Q. were completely oxidized by the tissues of the rest of the body. There is no significant difference between the R.Q.'s of the expired air of the meat-fed and starved animals. Crandall, Ivy and Ehni concluded, on the basis of urinary output, that 95% of the ketones produced by the livers of their starved and fat-fed animals was oxidized by other tissues of the body.

It is possible that some of the expired air R.Q.'s are higher than would be expected in a meat-fed or starved dog. The value of 1.0 in experiment 32 is undoubtedly too high. The minute volumes are, with the exception of experiment 32, somewhat higher than would be expected in a dog. This could, of course, account for the high R.Q. due to "blowing-off" of carbon dioxide. During the course of the majority of the experiments it was observed that the animals tended to exhibit a rather rapid, panting type of respiration, and frequently it was observed that the carbon dioxide capacity of the portal blood fell slightly between consecutive determinations. Even if it was assumed that the true expired air R.Q.'s were on a somewhat lower level the general relationship between the liver and expired air quotients would not be affected qualitatively.

## CONCLUSIONS

1. A method has been developed by which consistent determinations of the R.Q. of the liver in vivo can be made.

2. The R.Q. of the liver was found to have a value greater than 0.70 and to be not significantly different from the R.Q. of the entire animal when previously fed a meat diet.

3. The R.Q. of the liver was found to be lower than 0.70 in animals previously starved or fed a high fat diet.

LITERATURE CITED

BAUER, W., H. H. DALE, L. T. POULSSON AND D. W. RICHARDS 1932 The control of circulation through the liver. J. Physiol., vol. 74, p. 343.

BEST, C. H., G. C. FERGUSON AND J. M. HERSHEY 1933 Choline and liver fat in diabetic dogs. J. Physiol., vol. 79, p. 94.

BIALOCK, A., AND M. F. MASON 1936 Observations on the blood flow and gaseous metabolism of the liver of unanesthetized dogs. Am. J. Physiol., vol. 117, p. 328.

BLIXENKRONE-MØLLER, N. 1938 Respiratorischer Stoffwechsel und Ketonbildung der Leber. Z. f. Physiol. Chemie, vol. 252, p. 117.

CAMERON, G. R., AND B. T. MAYES 1930 Ligation of the hepatic artery. J. Path. and Bact., vol. 33, p. 799.

COLLENS, W. S., D. H. SHELLING AND C. S. BYRON 1927 Studies on the physiology of the liver. II. Effect of adrenalin upon blood sugar following ligation of the hepatic artery. Am. J. Physiol., vol. 79, p. 689.

CRANDALL, L. A., JR., H. B. IVY AND G. J. EHNI 1940 Hepatic acetone body production in the dog during fasting and fat feeding. Am. J. Physiol., vol. 131, p. 10.

CRANDALL, L. A., JR., AND G. M. ROBERTS 1936 Increased water exchange following Eck fistula in dogs. Am. J. Physiol., vol. 117, p. 318.

EDMUNDS, C. W. 1915 Some vasomotor reactions of the liver with special reference to the presence of vasomotor nerves to the portal vein. J. Pharm. and Exp. Therap., vol. 6, p. 569.

GEMMILL, C. L., AND E. G. HOLMES 1935 The formation of carbohydrate from fat in the liver of the rat. Biochem. J., vol. 29, p. 338.

HABERER, H. 1906 Experimentelle Unterbindung der Leberarterie. Arch. f. klin. Chir., vol. 78, p. 557.

HENDERSON, Y. 1918 Applications of gas analysis. IV. The Haldane gas analyzer. J. Biol. Chem., vol. 33, p. 31.

HIMWICH, H. E., AND W. B. CASTLE 1927 Studies in the metabolism of muscle. I. The respiratory quotient of resting muscle. Am. J. Physiol., vol. 83, p. 92.

LEATHES, J. B., AND H. S. RAPER 1925 The Fats. Longmans, Green and Co., London.

MODELL, W, 1939 Chlorazol fast pink BKS as an anti-coagulant. Sci., vol. 89, p. 349.

NARATH, A. 1916 Über Entstehung der anämischen Lebernekrose nach Unterbindung der Arteria Hepatica und ihre Verhütung durch arterioportale Anastomose. Deut. Z. f. Chir., vol. 135, p. 305.

SMITH, H. P., AND G. H. WHIPPLE  1930  Bile salt metabolism. IX. Eck fistula modifies bile salt output. J. Biol. Chem., vol. 89, p. 739.

STADIE, W. C., J. A. ZAPP, JR., AND F. D. W. LUKENS  1940  The effect of insulin upon the ketone metabolism of normal and diabetic cats. J. Biol. Chem., vol. 132, p. 423.

———————  1941  Intermediary metabolism in diabetes mellitus. On the synthesis of carbohydrate from fat in the liver and from acetoacetate in the kidney. J. Biol. Chem., vol. 137, p. 63.

VAN SLYKE, D. D., AND R. FITZ  1917  Studies of acidosis. VIII. The determination of B-hydroxybutyric acid, acetoacetic acid, and acetone in blood. J. Biol. Chem., vol. 32, p. 495.

VAN SLYKE, D. D., AND J. M. NEILL  1924  The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. J. Biol. Chem., vol. 61, p. 523.

# SENSORY NEURON DEGENERATION IN PIGS

## IV. PROTECTION AFFORDED BY CALCIUM PANTOTHENATE AND PYRIDOXINE [1, 2]

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SIX FIGURES

In previous reports (Wintrobe et al., '38, '40, '42), abnormal gait and degenerative changes in the nervous system of pigs have been described. It was pointed out that these lesions occurred as the result of a dietary lack of some substance present in whole liver and, less consistently, in brewers' yeast. In past experiments in this series it was shown that this disorder developed in animals fed diets containing adequate amounts of thiamine, riboflavin, nicotinic acid, wheat germ oil, and cod liver oil, as well as adequate protein, carbohydrate, fat and minerals.

In an attempt to discover the nature of the deficiency which leads to the development of the changes in the nervous system,

experiments were planned with the object of producing deficiencies of single vitamins. This appeared possible when pyridoxine and pantothenic acid became available in sufficient quantities, for it then was found in preliminary observations that good growth occurred and excellent health was maintained in pigs fed our basal diet together with these two vitamins in addition to thiamine, riboflavin, nicotinic acid, choline and cod liver oil.

The present report describes the effects produced when, of these vitamins, the following were *not* furnished in crystalline form in concurrent experiments with comparable groups of animals: group (1) thiamine; (2) riboflavin; (3) nicotinic acid; (4) pyridoxine; (5) choline; (6) calcium pantothenate; and (7) vitamin A. In several groups two or more vitamins were not furnished, as follows: group (8) pyridoxine and choline; (9) choline and calcium pantothenate; and (10) pyridoxine, choline, and calcium pantothenate. There were two control groups; namely, group (11) in which none of these vitamins was omitted, and group (11 a) in which none of these vitamins was omitted but highly purified ("vitamin free") casein was fed instead of the "crude" casein given the remainder of the animals. In a final group (12) desiccated whole liver was furnished instead of the above six "B" vitamins.

It is to be noted that with the exception of group (11 a) the animals received in their basal diet a grade of casein which cannot be regarded as vitamin free. According to our assays, made by the microbiological technique (Strong et al., '41), this casein contained approximately 400 μg. riboflavin per 100 gm. In view of the observations to be described it is possible that it furnished some nicotinic acid and vitamin A, although we have no information as to the actual amounts of these two vitamins which may be carried in the casein. Evidence will be cited which indicates that it furnishes insufficient nicotinic acid to permit good growth under certain conditions. Whatever the content of nicotinic acid, vitamin A and other vitamins such as biotin and inositol in our basal

diet may be, it is clear from the observations to be described that little thiamine, pyridoxine or pantothenic acid was available to the pigs other than as given in crystalline form.

A review of the literature was presented in a previous report (Wintrobe, Miller and Lisco, '40).

### GENERAL PROCEDURE

Included in this report are observations on two large lots of animals, those numbered from 6–29 to 6–50, inclusive (experiment VI) having been begun on the experiment in October, 1940, and those numbered from 6–51 to 6–80, inclusive (experiment VII) having been started in March, 1941.

The animals were received at our laboratory shortly after weaning at an age of approximately 3 weeks. The feeding, housing, and observation of the pigs were similar to that described elsewhere (Wintrobe, Miller and Lisco, '40). From the time of their arrival until the termination of the experiment their basal diet had the following percentage composition: "New Process" casein [6], 26.1; sucrose, 57.7; lard, 11.0; and swine salt mixture no. 3 (Wintrobe, Miller and Lisco, '40), 5.2.

During the first 4–5 months of each of the experiments each animal was fed 36.4 gm. of this diet per kilogram body weight per day. After the fifth month this was gradually reduced to one-half. The reduction in caloric intake was associated with no appreciable reduction in weight gain.

The basal diet was supplemented with cod liver oil [7] (0.5 gm. per kilogram body weight daily) except in group 7, which received viosterol [7] diluted in corn oil in amounts furnishing 87.5 international units vitamin D per kilogram body weight.

All animals were initially fed 3 gm. of brewers' yeast [7] per kilogram body weight per day. In experiment VI (animals numbered from 6–29 to 6–50, inclusive), a progressive reduction in the yeast supplement was begun when the pigs were 90–96 days of age, the yeast being entirely omitted when the animals were 104–110 days old. Vitamin supplementation as

[6] Sheffield By-Products Co.
[7] Mead Johnson and Company.

indicated in table 1 was begun at 97 to 103 days of age. In experiment VII (pigs 6–51 to 6–80, inclusive), the reduction of yeast was begun when the pigs were 73–75 days old and its omission was completed when they were 87–89 days of age. In this experiment the vitamin supplementation was begun at 80–82 days of age. In experiment VII the animals were placed on wire screens from the age of 5 months in an effort to prevent coprophagy.

TABLE 1

*Nature and quantity of vitamin supplements fed each experimental group.*

| SUPPLEMENT [1] | GROUP NUMBER | | | | | | | | | | | | | AMOUNT FED KG./BODY WEIGHT/DAY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 11a[2] | 12[3] | |
| Thiamine hydrochloride | | + | + | + | + | + | + | + | + | + | + | + | | 0.52 mg.[4] |
| Riboflavin | + | | + | + | + | + | + | + | + | + | + | + | | 0.12 mg. |
| Nicotinic acid | + | + | | + | + | + | + | + | + | + | + | + | | 1.20 mg. |
| Pyridoxine hydrochloride | + | + | + | | + | + | + | | + | | + | + | | 0.20 mg. |
| Choline chloride | + | + | + | + | | + | + | | | | + | + | | 10.00 mg. |
| Calcium pantothenate | + | + | + | + | + | | + | + | | | + | + | | 0.50 mg. |
| Vitamin A | + | + | + | + | + | + | | + | + | + | + | + | + | 900 I.U. |
| Vitamin D | + | + | + | + | + | + | + | + | + | + | + | + | + | 87.5 I.U. |

[1] The vitamins were fed by capsule every other day. The actual amount given was revised every 2 weeks according to the most recently recorded weight of each animal. In giving the vitamins, the capsule was placed far back in the animal's oropharynx. The pig was then observed for 5 or 10 minutes, and, if regurgitation of the capsule occurred, a second one was given.

[2] Group 11a was given acid, alkali and alcohol-washed casein instead of the less refined product fed the rest of the animals.

[3] Group 12 was given desiccated whole liver, 1.5 gm. per kilogram body weight daily, in the place of crystalline vitamins.

[4] This dosage was reduced to 0.13 mg. per kilogram per day in pigs numbered 6-51 to 6-80, inclusive, at 130–140 days of age.

The preliminary feeding of yeast was carried out in order to ensure satisfactory weaning and to allow the more gradual development of vitamin deficiency than would result if the animals were placed on the experimental regime at once.

All the animals were autopsied and blocks of all the tissues placed in formalin. Microscopic examinations were made of all the organs except the brain and eyes from one animal in each of the thirteen groups. If pathological alterations were noted in any of these, similar tissues from the other animals

in the group were examined. Because of our special interest in the nervous system, the brachial and sciatic nerves, spinal ganglia and spinal cord of every animal were examined. The brain and eyes were saved for study.

## OBSERVATIONS

Details of the general observations do not come within the scope of this report but a few need mention.

*Growth.* Growth curves for all the animals appear in figure 1. It is obvious that none of our animals quite equalled the rate of growth of comparable pigs on a well-balanced diet of crude foodstuffs. Good growth was achieved, however, in those pigs receiving six "B" vitamins in addition to cod liver oil (groups 11 and 11 a), and in the animals receiving no crystalline nicotinic acid (group 3), no vitamin A (group 7), no added choline (group 5) and no crystalline riboflavin (group 2). Pantothenic acid deficiency, whether alone (group 6) or in combination with a lack of choline (group 9) or with pyridoxine and choline deficiency (group 10), produced the most marked disturbance of growth. Pyridoxine deficiency also reduced the rate of growth (groups 4 and 8) but not to the degree produced by pantothenic acid deficiency.

*Appearance of animals.* Malnutrition in the pig is usually manifested first by an appearance of untidiness which is in marked contrast with the well-groomed, clean-looking healthy pig. In pigs given no pyridoxine (group 4) or calcium pantothenate (group 6) and in those deficient in both of these vitamins (groups 8, 9 and 10) the coats often appeared thin and dry. In those deficient in pantothenic acid the alopecia was sometimes quite marked and was accompanied by some reddening of the skin. Thinning of the hair was most noticeable over the rumps and along the spinal column. Failure to supplement the basal diet with choline (group 5) seemed to bear little relation to the appearance of the animals and those given no added riboflavin (group 2) or nicotinic acid (group 3) appeared in excellent condition throughout the experiment. The omission of vitamin A from the supplement
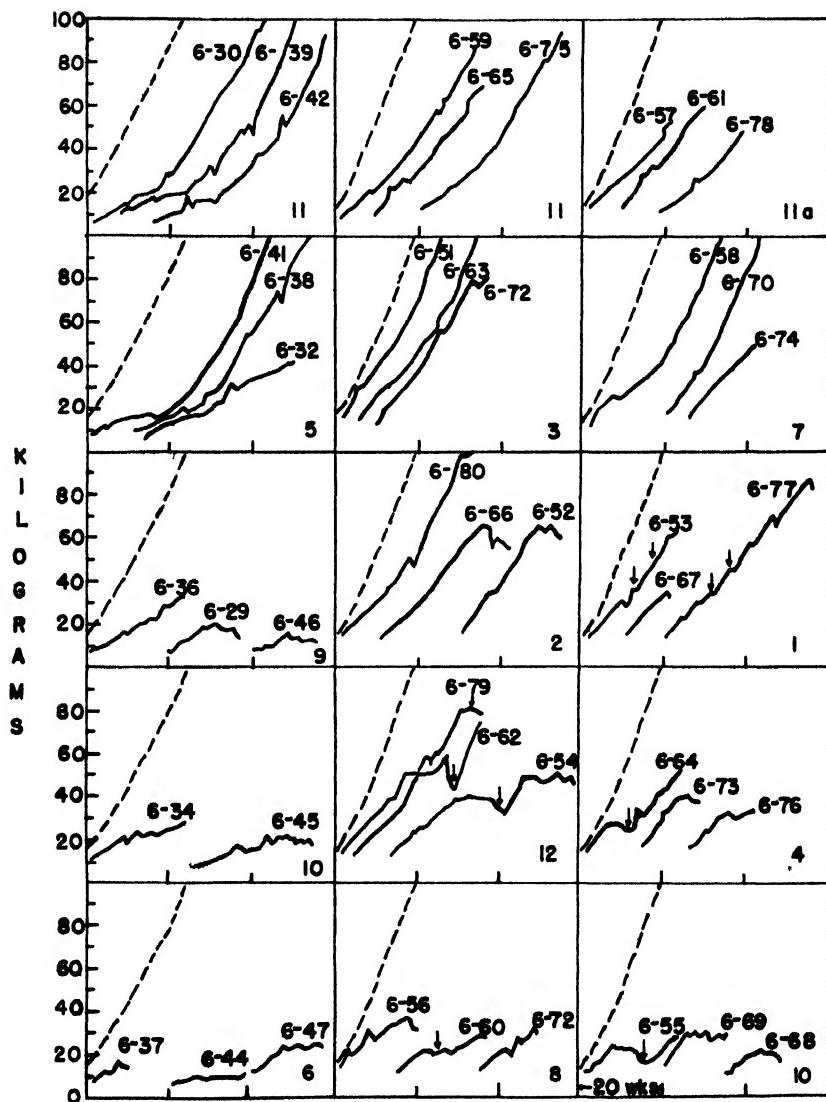
Fig. 1 Growth curves of animals in the different experimental groups as described in table 1. All curves begin at the time when crystalline vitamin supplements were added to the basal diet. The broken line represents the growth of pigs given a mixed diet at the Beltsville Research Center and fed and handled according to record of performance procedure.

(group 7) was not associated with any changes in the hair or skin of the affected animals.

*Alimentary system.* Loss of appetite and vomiting were the first signs of deficiency to appear in the pigs which received no thiamine (group 1), and were quickly succeeded by other more serious signs. As in the thiamine-deficient pigs vomiting was encountered in all the animals given desiccated liver (group 12) instead of synthetic vitamins. This symptom was usually preceded or accompanied by anorexia. In both these groups these two signs could not be correlated with any pathological changes. That they were closely related to changes in the blood pyruvic acid level, and were the result of thiamine deficiency is supported by the fact that the appetite returned and vomiting ceased when thiamine in sufficient dosage was administered. These and other observations in animals deficient in thiamine will be reported separately (Wintrobe, Stein, Miller, Follis, Najjar, and Humphreys, '42; Follis, Miller, Wintrobe and Stein, '42).

Anorexia also occurred in animals receiving neither pyridoxine (groups 4 and 8), calcium pantothenate (groups 6 and 9), nor either vitamin (group 10), but in these pigs it appeared as a late phenomenon of deficiency and was only rarely accompanied by vomiting.

Glossitis was not observed in any of the animals. Ulceration was found irregularly in the stomach but its incidence seemed more related to that of trichobezoars than to any form of nutritional deficiency.

Diarrhea occurred with frequency whenever the vitamin supplement failed to include calcium pantothenate (groups 6, 9 and 10) or, less regularly, when pyridoxine was lacking (groups 4, 8 and 10). Only in uncomplicated pantothenic acid deficiency (group 6), were changes in the bowel noted. The pathological change was confined to the colon, the process consisting of widespread, patchy injection of the mucosal surface of the entire large bowel. Microscopically there was necrosis of the superficial portion of the mucosa with leuko-

cytic infiltration of the remaining mucous membrane and submucosa. Numerous glands were dilated and their lumens filled with leukocytes (fig. 2).

*Nervous system. Abnormal gait and sensory neuron degeneration (figs. 3, 4 and 5).* The abnormal gait that occurred in these experiments was of the same type as that previously observed (Wintrobe et al., '40, '42). The affected animal at first shows only a slightly high lift of the hind legs in walking
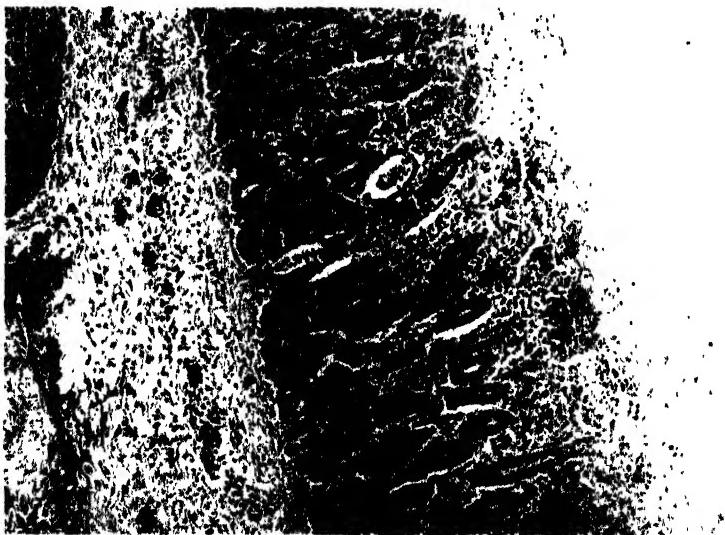


Fig. 2   Photomicrograph of mucosa of colon of a pig deficient in pantothenic acid (6-37) showing necrosis of the superficial layer of the mucosa with leukocytic infiltration of the remainder. There are perivascular accumulations of cells about the blood vessels in the submucosa.   × 90.

and there is some swaying of the hind quarters. As the condition progresses a definite "goose step" develops, and the animal stumbles and falls repeatedly. When ataxia has become marked, the hind legs assume grotesque positions even when the pig is at rest. In only a few animals has the abnormality involved the forelegs and then only long after the characteristic gait had affected the hind quarters.
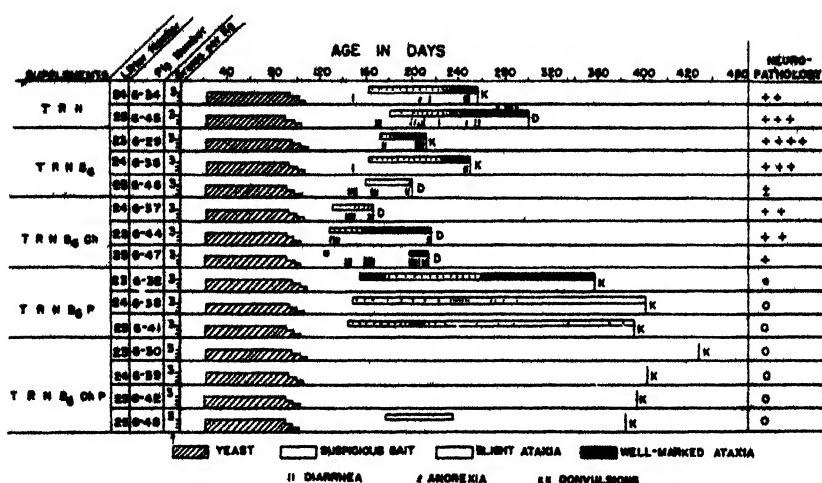
Fig. 3 Experiment VI. The development of abnormal gait, convulsions, loss of appetite and diarrhea, and the extent of neuropathological changes in pigs given a diet low in water-soluble vitamins other than as supplied in the form of the pure, synthetic vitamins indicated. All the pigs received the same basal diet. Vitamin supplements were started when the quantity of yeast was reduced below 3 gm. per kilogram and were continued throughout the life of the pigs.

Note the freedom from any symptoms of deficiency whatsoever in the pigs given thiamine (T), riboflavin (R), nicotinic acid (N), pyridoxine (B₆), choline (Ch) and calcium pantothenate (P), and the development of abnormal gait and neuropathological changes when pantothenic acid or pantothenic acid as well as pyridoxine was lacking.

Convulsions were observed only in the animals not given pyridoxine.

Abnormal gait, as described in the text, was observed in all the pigs given no choline, but neuropathological changes were found only in one animal. These were of a type not seen heretofore and are therefore marked *.

D indicates that the animal died, K that it was sacrificed.

The scoring of neuropathological changes is that described in the footnote to table 2.

Abnormal gait as well as sensory neuron degeneration occurred in all pigs failing to receive either pyridoxine (group 4) or calcium pantothenate (group 6), alone or in certain combinations involving also a lack of choline (pyridoxine and choline, group 8; calcium pantothenate and choline, group 9; pyridoxine, calcium pantothenate and choline, group 10). Abnormal gait was not observed in pig 6–71 of group 8, an animal which died after repeated convulsions had taken place during

TABLE 2

*Data concerning incidence of abnormal gait, treatment and pathological changes in all animals.*

| GROUP NO. AND VITAMIN SUPPLEMENT [1] | PIG NO. AND SEX | AGE SUPPLEMENT STARTED | AGE ATAXIA OBSERVED | AGE AND MANNER OF DEATH [3] | Nervous system [4] | Other tissues |
|---|---|---|---|---|---|---|
| | | *days* | *days* | | | |
| 1 RNB₆ChP (no. T) | 6-53 M | 87 [2] | ...... | 246 D | 0 | Focal necroses, myocardium; Hemorrhage and edema, lung |
| | 6-67 F | 87 | ...... | 171 D | 0 | Focal necroses, myocardium; Edema, lung |
| | 6-77 F | 87 [2] | ...... | 325 D | 0 | Focal necroses and scars, myocardium; Edema, lung |
| 2 TNB₆ChP (no R) | 6-52 M | 87 | ...... | 260 K | 0 | Ulcer, tongue; abscesses, l. hind leg and between vertebrae. Adhesions, pericardium and pleurae |
| | 6-66 F | 87 | ...... | 312 K | 0 | Abscesses, both hind legs |
| | 6-80 F | 87 | ...... | 314 K | 0 | ................ |
| 3 TRB₆ChP (no N) | 6-51 M | 87 | ...... | 289 K | 0 | Healed ulcer, stomach |
| | 6-63 M | 88 | ...... | 292 K | 0 | ................ |
| | 6-72 F | 88 | ...... | 285 K | 0 | Small abscesses, kidney |
| 4 TRNChP (no B₆) | 6-64 M [9] | 87 | 137 [2] | 259 K | + | Ulcer, stomach; Slight hemosiderosis, spleen |
| | 6-73 F [9] | 87 | 152 | 197 D | + [5] | Autolysis; hemosiderosis, spleen, liver and marrow; fatty liver, mod. |
| | 6-76 F [9] | 88 | 141 | 202 K | ++ | Fatty liver, mod.; hemosiderosis, spleen, liver and marrow |
| 5 TRNB₆P (no Ch) | 6-32 F | 110 | 270 | 357 K | Lat.[6] | Fatty liver, marked |
| | 6-38 F | 107 | 232(270)[7] | 402 K | 0 | Fatty liver, marked; ulcer, stomach; adhesions, pleura |
| | 6-41 M | 106 | 198(228)[7] | 390 K | 0 | Fatty liver, marked |
| 6 TRNB₆Ch (no P) | 6-37 M | 107 | 142(?)[8] | 166 D | ++ | Colitis |
| | 6-44 M | 104 | 145 | 217 D | ++ | Colitis |
| | 6-47 F | 104 | 201 | 214 D | +++ | Colitis |
| 7 TRNB₆ChP (no A) | 6-58 F | 87 | 302 | 342 K | 0 | Healed fractures, ribs |
| | 6-70 M | 87 | 290 | 315 K | 0 | Focal necroses, voluntary muscle |
| | 6-74 F | 87 | ...... | 205 D | 0 | ................ |
| 8 TRNP (no. B₆Ch) | 6-56 F [9] | 88 | 205 | 229 K | + | Fatty liver, mod.; hemosiderosis, spleen, liver and marrow |
| | 6-60 M [9] | 87 | 149 [2] | 242 K | ++++ | Fatty liver, sl.; hemorrhage, stomach; Hemosiderosis, spleen, liver and marrow; Gen. caseous lymphadenopathy |
| | 6-71 M [9] | 87 | ...... | 187 D | ± | Fatty liver, marked; hemorrhage, stomach; subarachnoid hemorrhage; Hemosiderosis, spleen, liver and mar. |
| 9 TRNB₆ (no ChP) | 6-29 F | 110 | 183 | 211 K | ++++ | Abscesses, skin |
| | 6-36 M | 107 | 224 | 240 K | +++ | Fatty liver, slight |
| | 6-46 M | 106 | 199 | 200 D | ± | Lobular pneumonia |

TABLE 2 — (*Continued*)

| GROUP NO. AND VITAMIN SUPPLEMENT[1] | PIG NO. AND SEX | AGE SUPPLEMENT STARTED | AGE ATAXIA OBSERVED | AGE AND MANNER OF DEATH[3] | PATHOLOGICAL CHANGES Nervous system[4] | Other tissues |
|---|---|---|---|---|---|---|
| | | *days* | *days* | | | |
| 10 TRN (no B₆ChP) | 6-34 M | 107 | 238 | 257 K | ++ | Hematomata, both hams; hemosiderosis, spleen, liver and b. m. Fatty liver, extreme |
| | 6-45 M [2] | 104 | 255 | 300 D | +++ | Fatty liver, moderate Hemosiderosis, spleen and marrow |
| | 6-55 F [2] | 87 | 158 [2] | 256 K | +++ | Pleural and pericardial adhesions Fatty liver, moderate |
| | 6-68 F | 87 | 151 [2] | 189 D | +++ | Fibrinous pericarditis; hemosiderosis, spleen and marrow; fatty liver, sl. |
| | 6-69 M | 87 | 135 [2] | 198 D | +++ | Lobular pneumonia; hemosiderosis, spleen and marrow; fibrinous pleurisy; fatty liver, moderate |
| 11 TRNB₆ChP | 6-30 F | 106 | ...... | 448 K | 0 | Postpartum uterus |
| | 6-39 F | 106 | ...... | 402 K | 0 | Pregnant, 7 fetuses Ulcer, stomach |
| | 6-42 M | 106 | ...... | 403 K | 0 | Abscesses, skin |
| | 6-59 F | 87 | ...... | 328 K | 0 | ............... |
| | 6-65 M | 88 | ...... | 282 D | 0 | Acute gastritis |
| | 6-75 F | 87 | ...... | 328 K | 0 | ................ |
| | 6-48 F | 91 | ...... | 320 K | 0 | ................ |
| 11a TRNB₆ChP (Vit.-free casein) | 6-57 F | 88 | ...... | 236 K | 0 | ............... |
| | 6-61 M | 87 | ...... | 236 K | 0 | ............... |
| | 6-78 F | 87 | ...... | 238 K | 0 | ................ |
| 12 Des. liver (low T) | 6-54 F | 87 [2] | ...... | 410 D | 0 | Focal necroses, myocardium Edema, lung |
| | 6-62 M | 88 [2] | ...... | 327 K | 0 | ............... |
| | 6-79 F | 87 [2] | ...... | 319 K | 0 | Healed fracture, vertebra |

[1] T = thiamine, R = riboflavin, N = nicotinic acid, B₆ = pyridoxine, Ch = choline, P = calcium pantothenate. For purposes of ready reference the supplement not furnished is shown in brackets.

[2] Pigs 6-53, 6-77, 6-54, 6-62 and 6-79 were treated with thiamine from time to time. Treatment with pyridoxine, as described in the text, was commenced in the following pigs at the age shown in parentheses: 6-64 (157); 6-60 (174); 6-55 (187); 6-68 (181); 6-69 (185).

[3] K = killed; D = died.

[4] 0 = no lesions; + = definite changes in the peripheral nerves only: these consisted in myelin degeneration with free fat granules and swollen neurilemmal cells (when there was less extensive change in the peripheral nerves with only Marchi degeneration demonstrable, the lesion was graded as ±); ++ = changes were accompanied by chromatolysis in the cells of the posterior root ganglia; +++ = degeneration of the posterior roots and the root entry zone of the spinal cord, in addition to the ganglion cell and peripheral nerve changes already noted; ++++ = conspicuous degenerative changes in the nerves, ganglia, roots and posterior funiculi of the spinal cord.

[5] Tissues badly autolysed; cord and ganglion cell changes could not be studied.

[6] Degenerative changes in the lateral funiculi of the spinal cord and nowhere else.

[7] Pigs 6-38 and 6-41 were no longer ataxic after the day indicated in parentheses.

[8] Gait appeared mildly ataxic only on the 142nd day.

[9] Epileptiform convulsions were observed in the following animals, commencing on the days indicated in parentheses: 6-64 (157); 6-73 (157); 6-76 (165); 6-56 (203); 6-60 (154); 6-71 (158); 6-45 (273); 6-55 (168). Three of these were treated with pyridoxine and the convulsions did not reappear.

his third month on the experiment. At autopsy, however, even in this pig there were found early degenerative changes in the sciatic nerve in addition to a small subarachnoid hemorrhage. The latter probably developed following a convulsion and may have caused the animal's death.

Slightly abnormal gait was also seen in all of the animals given no supplement of choline (group 5), but was persistent in only one of the three (pig 6–32). In the other two members of this group the gait became normal again later in the experiment (see fig. 3 and table 2). The ataxia in these animals differed somewhat from that which we have seen previously inasmuch as the usual "goose step" and "spasticity" of the
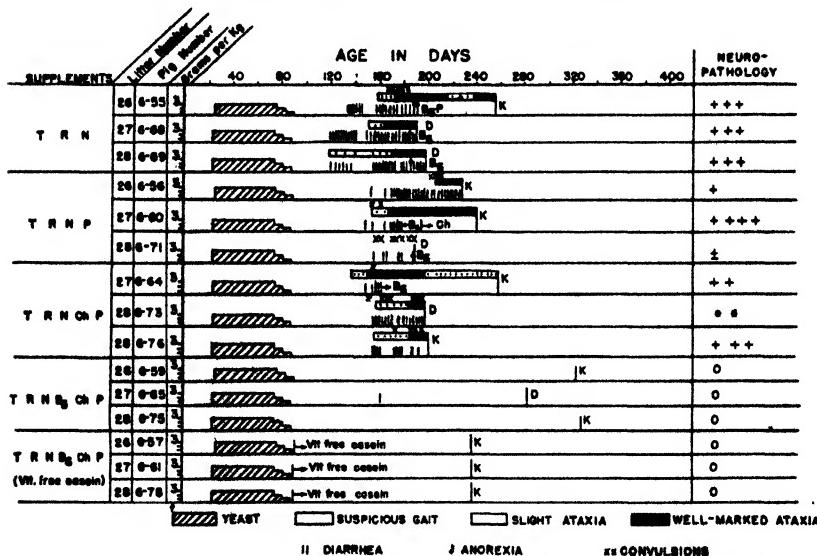


Fig. 4 Experiment VII. Legends same as in figure 3. Vitamin supplements were started when yeast was stopped.

Note the freedom from any symptoms of deficiency in pigs given thiamine, riboflavin, nicotinic acid, pyridoxine, choline and calcium pantothenate, even when "vitamin-free" casein was fed instead of "crude" casein.

Abnormal gait and neuropathological changes developed when either pyridoxine or pyridoxine as well as pantothenic acid was lacking.

Convulsions were observed only in the animals not given pyridoxine.

Arrows pointing vertically represent single injections of pyridoxine (B$_6$) or calcium pantothenate (P), as indicated. Arrows pointing horizontally refer to daily administration.

hind legs were not observed. The gait had an unbalanced, staggering quality. Changes in the nervous system were seen only in pig 6–32. These consisted of myelin degeneration without gliosis and were confined to the lateral funiculi of the spinal cord. No alterations in the ganglia, peripheral nerves, or other spinal tracts were seen.

Two of the pigs in group 7 receiving no supplement of vitamin A, developed abnormal gaits quite late in the experiment. The animals exhibited chiefly a peculiar staggering manner of locomotion. In none of these animals were neuropathological changes noted, but foci of necrosis in the striated muscle, very similar to those described in experimental vitamin E deficiency in guinea pigs and rabbits (Mackenzie, Levine and McCollum, '40) were seen in one animal (6-70). These lesions were of only rare occurrence, however, and their significance is doubtful.

Neither abnormalities of gait nor histologic changes in the nervous system were observed in the animals given no crystalline supplement of either thiamine (group 1), riboflavin (group 2) or nicotinic acid (group 3).

*Effect of treatment.* Only five animals were treated after signs of deficiency had become apparent. Two of these succumbed within a few days after treatment was instituted (6-68, 6-69). Pig 6-55, which, like 6-68 and 6-69, had received neither pyridoxine, choline, nor calcium pantothenate, was given pyridoxine and calcium pantothenate in the usual maintenance dose (table 1) after it had become practically helpless from progressive ataxia of a month's duration. During the time that ataxia developed, anemia and inactivity had appeared and growth ceased. The improvement in the blood picture following treatment was dramatic. Growth, activity and appetite were resumed within 3 weeks. The gait improved considerably but more slowly and, when this animal was killed after 2 months of continuous treatment, the gait was still definitely but only mildly ataxic. Pig 6-60, in group 8, was given 110 mg. pyridoxine intravenously after ataxia had become progressively worse and had been present for at least

25 days. Treatment was continued orally with a daily dose of 200 μg. per kilogram body weight. Although the anemia which had concurrently developed disappeared rapidly, and convulsions ceased, the gait not only failed to improve but became worse. The remaining animal which received treatment (6-64) was given 10 mg. pyridoxine intravenously, following which 200 μg. per kilogram body weight was given orally each day. Ataxia had been present for 20 days and had progressed to a severe stage by the time therapy was begun.
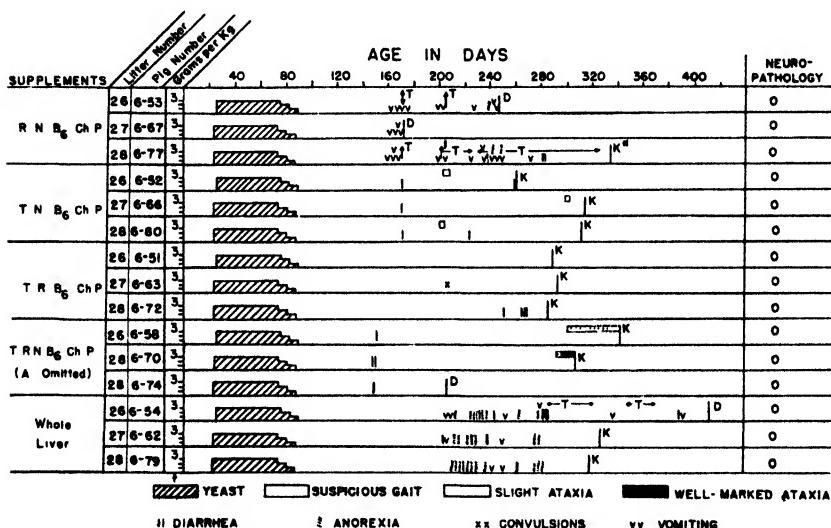


Fig. 5  Experiment VII, continued. Legends same as in figures 3 and 4.

Note the lack of neuropathological changes in animals given no crystalline thiamine, no riboflavin or no nicotinic acid. Pigs 6-58, 6-70 and 6-74 were given all the water soluble vitamins indicated but received no vitamin A. Although the gait became abnormal in two of these pigs, no neuropathological changes were observed.

Symptoms of nutritional deficiency, in the form of anorexia and vomiting were observed only in the pigs not given thiamine and in those fed desiccated whole liver, which contains little thiamine. Failure to develop signs of riboflavin deficiency was probably due to the content of this vitamin in the casein included in the basal diet. It is not known whether a similar explanation can be given for the failure to develop signs of nicotinic acid deficiency in the pigs given no crystalline nicotinic acid, and for the equivocal results in the animals given no supplement of vitamin A.

There was marked though gradual improvement in the gait over a period of 2 to 2½ months. As in the other treated animals, convulsions and anemia disappeared promptly and growth was resumed.

This number of observations is too small to allow conclusions to be drawn in regard to the effects of therapy. Further studies are in progress.

*Convulsions.* Epileptiform fits (Wintrobe et al., '42) occurred in various pigs which did not receive pyridoxine. These will be discussed in a separate report (Wintrobe, Miller, Follis and Stein, '42).

*Anemia.* Anemia occurred in all the animals whose supplement did not include pyridoxine. Marked hemosiderosis of the spleen, and to a lesser extent of the liver and bone marrow, was found in the pyridoxine deficient animals which were not treated. Details concerning the anemia and its response to treatment with this vitamin will appear in another report (Wintrobe, Follis, Miller, Stein, Humphreys and Suksta, '42).

*Incidence of infection.* Localized ulcerating infections occurred on the extremities of several pigs. These seemed to follow minor trauma, such as cuts and abrasions. Many fibrous adhesions were found in the pleural, pericardial and peritoneal cavities of those pigs which did not receive either pyridoxine, choline, or calcium pantothenate in experiment VII (see group 10, table 2). This was probably the result of some healed infection rather than the specific effect of deficiency, since such changes were not observed in comparable animals in experiment VI. Similar changes were noted in the pleura and pericardium of pigs 6-52 (group 2) and 6-38 (group 5).

*Liver.* An increased amount of fat in the liver (fig. 6) was found in animals given no supplement of either choline (group 5), pyridoxine or choline (group 8), or pyridoxine, choline or calcium pantothenate (group 10). However, a slight amount of fat was found in only one animal (6-36) of the group failing to receive either choline or calcium pantothenate (group 9). It is noteworthy, also, that fatty infiltration was

seen in pigs given choline but not pyridoxine (group 4), with the exception of the one animal (6-64) of this group which had been treated with pyridoxine. It would thus appear that fatty infiltration of the liver may arise when the diet lacks either choline, or pyridoxine, or both. When pantothenic acid was lacking in addition to choline, however, little or no fatty infiltration was found.
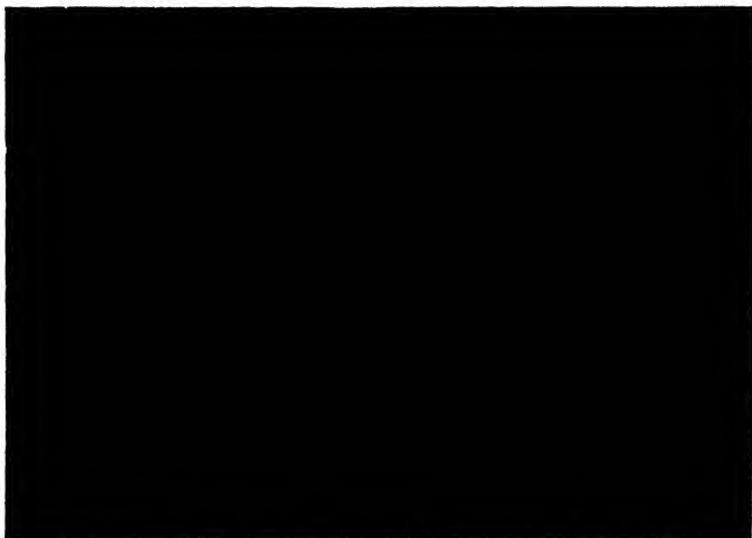


Fig. 6   Photomicrograph of liver of a pig not given choline (6-38), showing extremely fatty infiltration of the central and mid-zonal portions of the lobules. There is no cirrhosis, the connective tissue shown being normal for the pig.  × 45.

*Adrenals.* In animals not given calcium pantothenate as a supplement (groups 6, 9 and 10) neither gross nor microscopic changes were found in the adrenals.

*Gonads.* Varying degrees of degeneration of the seminiferous tubules were observed in all animals in which poor growth occurred. On the other hand, gestation occurred in two of the pigs fed all six crystalline vitamins. In one of these animals (6-39) seven fetuses were unexpectedly found in the uterus at autopsy, while the second (6-30) gave birth to two pigs. One was found dead and the second died several hours after birth.

## DISCUSSION

In table 3 are summarized the results of our studies involving the use of crystalline vitamins, as described in the present report and in earlier ones (Wintrobe et al., '38, '40), as well as those dealing with crude substances (Wintrobe et al., '42). It can be seen that sensory neuron degeneration developed in forty-one pigs when either pyridoxine or pantothenic acid, or both, were lacking; conversely, we have not observed such changes in the nervous system in any of twenty-six animals when both of these vitamins were furnished.

The basal diet, which included crude casein, sugar, lard, a salt mixture and cod liver oil, did not in itself furnish protective substances since all of the animals mentioned above as developing neurological changes were fed the same basal diet. Furthermore, the possible presence in crude casein of protective factors is ruled out by the fact that neurological changes did not occur when a purified casein was substituted for crude casein in the diets of animals receiving pyridoxine and calcium pantothenate in addition to thiamine, riboflavin, nicotinic acid and choline.

The conclusion that pyridoxine and pantothenic acid are necessary in maintaining the integrity of the nervous system in the pig is consistent with our earlier observations concerning the role of crude substances (table 3). Whole liver is an excellent source of both of these substances and different brands of yeast contain 30 to 75 µg. of pyridoxine per gram and 30 to 200 µg. of pantothenic acid.

In table 4 is given the amount of pantothenic acid in various fractions of liver, together with a roughly graded comparison of the potency of each fraction in preventing sensory neuron degeneration as described by us recently (Wintrobe et al., '42). The figures for pantothenic acid were obtained by the microbiological technique (Pennington, Snell and Williams, '40). An unsuccessful attempt was made to determine the pyridoxine content of these fractions by a chemical method (Scudi, '41).

TABLE 3

Summary of observations on nutritional deficiencies in pigs.[9]

| SUPPLEMENT GIVEN * | NO. OF PIGS | GROWTH | ANOREXIA OR VOMITING | DIARRHEA | HAIR UNTIDY OR THIN | CONVULSIONS | ANEMIA | NEUROPATHOLOGY 0 | + | ++ | +++ | ++++ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T | 3 | ± | + | + | + | 0 | ++ | — | — | 2 | 1 | — |
| TR | 3 | ± | + | ++ | + | 0 | + | — | — | 1 | 1 | 2 |
| TN | 3 | + | + | + | + | 0 | ++ | — | — | 2 | 6 | 5 |
| TRN [1] | 20 | + | + | + | ++ | + | + | — | 1 | 9 | 1 | 1 |
| TRNB$_3$ | 3 | + | + | + | + | 0 | 0 | — | — | — | 1 | — |
| TRNP | 3 | + | + | + | ++ | + | + | — | 2 | 2 | 1 | 1 |
| TRNB$_3$Ch | 3 | + | + | + | + | + | + | — | 1[2] | 1 | 1 | — |
| TRNChP | 3 | ++ | + | + | + | + | 0 | — | 1[3] | — | 1 | — |
| TRNB$_3$P | 3 | ++ | 0 | 0 | 0 | 0 | + | 2 | — | — | — | — |
| TRB$_3$ChP | 3 | + | 0 | 0 | 0 | 0 | 0[4] | 3 | — | — | — | — |
| TNB$_3$ChP | 3 | +++ | + | 0 | 0 | 0 | 0 | 3 | — | — | — | — |
| RNB$_3$ChP | 3 | + | 0 | 0 | 0 | 0 | 0 | 3 | — | — | — | — |
| TRNB$_3$ChP [5] | 17 | + | 0 | 0 | 0 | 0 | 0 | 17 | — | — | — | — |
| Yeast, type N | 6 | + | 0 | 0 | 0 | 0 | 0 | 3 | — | 1 | 1 | 1 |
| Yeast, types N + F | 3 | + | + | 0 | 0 | 0 | 0 | — | 2 | 2 | 1 | — |
| Yeast, types N + M | 5 | + | 0 | 0 | 0 | 0 | 0 | 2 | — | — | — | — |
| Yeast, type M [6] | 3 | + | + | 0 | 0 | 0 | + | 5 | — | — | — | — |
| Whole liver | 3 | + | 0 | 0 | 0 | 0 | 0 | 3 | — | — | — | — |
| Whole liver + TRN [7] | 12 | + | 0 | 0 | + | 0 | + | 12 | 2 | 2 | 1 | 1 |
| "Press cake" [8] + TRN | 3 | ++ | 0 | + | + | + | + | — | 1 | 3 | 1 | — |
| "Whipple" fraction + TRN | 3 | ++ | + | + | + | + | + | — | — | — | — | — |
| "Cohn" fraction + TRN | 1 | + | 0 | 0 | + | 0 | ++ | — | — | 2 | — | — |
| "Parenteral" fraction + TRN | 3 | ++ | 0 | + | + | + | + | 1 | 2 | 1 | 1 | — |
| Permutit fraction + TRN | 3 | ++ | + | 0 | 0 | 0 | 0 | 1 | — | 2 | 2 | — |
| Mixture + TRN | 3 | + | 0 | 0 | 0 | 0 | + | 3 | — | — | 1 | — |
| | 118 | | | | | | | | | | | |

[1] Of these animals three were given wheat germ oil in addition to TRN.

[2] Tissues badly autolyzed; cord and ganglion cell changes could not be studied. Changes in the nerves were as severe as those in the other two pigs; therefore grading should probably be ++ or +++.

[3] Degenerative changes only in the lateral spinal funiculi.

[4] Anemia in these two groups was mild and related to infection.

[5] Three of these animals were not given vitamin A.

[6] Of these animals two were given wheat germ oil in addition to type "M" yeast. Wheat germ oil has been shown (Wintrobe, Miller and Lisco, '40) to afford no protection against sensory neuron degeneration.

[7] Of these animals, three were given wheat germ oil and type "M" yeast in addition to desiccated liver.

[8] This and the succeeding fractions of liver were given orally and are described fully elsewhere (Wintrobe et al., '42).

[9] It is to be noted that the chief object of these experiments has been to produce chronic deficiencies. Except in three animals described in the text, the basal diet included relatively crude casein rather than the "vitamin free" type. Furthermore, the supplement of yeast given all the pigs in their earlier weeks of life was usually removed only gradually as the supplement to be assayed was added to the diet.

* For meaning of various letters see footnote 1 to table 2.

It will be noted that the relatively high content of pantothenic acid in the "parenteral" and "Cohn" fractions of liver is consistent with the finding that these were superior to other fractions in preventing sensory neuron degeneration but were not as effective as whole liver.

Three brands of yeast were used in our earlier studies. Unfortunately assays for the content of pyridoxine and pantothenic acid in all three of these at the time they were used are not available. It is noteworthy, however, that all animals

TABLE 4

*Content of pantothenic acid in liver and liver fractions, with a rough comparison of their potency in protecting the pig from sensory neuron degeneration.*

|  | PANTOTHENIC ACID [1] | DEGREE OF PROTECTION [2] |
|---|---|---|
|  | $\mu g.$ |  |
| Desiccated whole liver | 213 | Complete |
| "Press cake" | 45 | Poor |
| "Whipple" fraction | 8.8 | Very poor |
| "Cohn" fraction | 76 | Fair |
| "Parenteral" liver extract | 82 | Good |
| "Permutit" fraction | 1.3 | Very poor |
| Mixture of "press cake," "Whipple," "parenteral," and "permutit" fractions | 137 | Complete |

[1] The figures represent the amount of vitamin found in that quantity of each substance derived from 5 gm. fresh liver.

[2] In grading the "degree of protection," the quantity of each fraction fed, the duration of time prior to the onset of ataxia, and the degree of neuropathology have been considered.

given type "M" yeast (see table 3) were protected and it seems plausible to assume that this yeast was superior quantitatively and perhaps qualitatively to type "N." "F" yeast was never used continuously in any group of animals.

Our observations in pigs give no support to the view that thiamine deficiency results in damage to the nervous system. Definite thiamine deficiency was produced in this experiment. Anorexia, vomiting and elevation in blood pyruvic acid occurred, as well as myocardial changes similar to but more extensive than those recently described in pigeons (Swank,

'40) and in dogs (Swank, Porter and Yeomans, '41) as resulting from thiamine deficiency. Although a deficiency state of 84 to 238 days' duration was produced, neurological lesions were not found in either the peripheral nerves, spinal ganglia or spinal cord of any of our animals.

The absence of all signs of nutritional deficiency in the pigs given no crystalline riboflavin (group 2) and in those given no nicotinic acid (group 3), as well as the equivocal results in those given no supplement of either choline (group 5) or vitamin A (group 7) makes it seem possible that these substances were supplied in sufficient amounts in the basal diet, particularly in the casein. According to assays of the casein, the pigs received as much as 40 μg. riboflavin per kilogram body weight through this medium. We have no adequate data regarding the content of the other above-mentioned vitamins in the basal diet. Consequently the results in respect to them must be regarded as inconclusive. In regard to choline it should be pointed out that the basal diet contained a high proportion of casein, thus making the conditions for the production of choline deficiency unfavorable.

In respect to nicotinic acid it should be pointed out that in earlier experiments in which the same type of casein was used as in the present series, when nicotinic acid was added to the same basal diet supplemented only with thiamine or with thiamine and riboflavin, striking improvement in rate of growth and in the condition of the pigs was observed (Wintrobe, Miller and Lisco, '40). Clearly the basal diet did not then furnish optimal amounts of nicotinic acid. Unless the content of this vitamin in casein varies from lot to lot, it would appear that the needs for nicotinic acid are different when only thiamine and riboflavin are furnished in crystalline form as compared with the requirements when pyridoxine, choline and calcium pantothenate are given as well.

### SUMMARY

1. Young pigs fed a ration of crude casein, lard, sucrose and a salt mixture, supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine, choline, calcium pantothenate and cod

liver oil developed normally and exhibited no changes in the nervous system during life or at autopsy.

2. Pigs similarly fed but receiving supplements of B vitamins which failed to include either calcium pantothenate or pyridoxine, developed an abnormal gait and showed degenerative changes in the peripheral nerves, the posterior root ganglia, the posterior roots, and the posterior funiculi of the spinal cord.

3. In the animals whose supplements did not contain calcium pantothenate, a subacute inflammation of the colon was found, in addition to the changes in the nervous system.

4. In the animals whose supplements did not contain pyridoxine, epileptiform convulsions and anemia were observed in addition to the above changes in the nervous system. Both the convulsions and the anemia disappeared promptly following the administration of pyridoxine.

5. When thiamine was not furnished with the vitamin supplements no changes in the nervous system were observed although other signs attributable to thiamine deficiency developed.

6. The omission of choline was associated with some abnormality of gait in all of three animals but lesions were observed in the nervous system of only one. These lesions, furthermore, differed from those observed in association with pyridoxine or pantothenic acid deficiency.

7. The results of observations on 118 pigs are summarized and the signs of chronic deficiency of various water soluble vitamins are presented.

8. It is concluded that both pyridoxine and pantothenic acid are necessary in maintaining the integrity of the nervous system in the pig under the conditions of these experiments.

Dr. Theodore L. Danforth, Jr. and Dr. Raul Alcayaga assisted in some of these studies and technical aid was given by Mr. Adolph Suksta, Mrs. Lottie Lowenstein, Mrs. Eleanor Collins, Miss Eva Hodgens, Miss Elaine Cohen, Miss Gertrude Merr and Mr. William Mock. Photomicrographs were made by Mr. Milton Kougl.

## LITERATURE CITED

FOLLIS, R. H., JR., M. H. MILLER, M. M. WINTROBE AND H. J. STEIN 1942 Development of myocardial necrosis and absence of nerve degeneration in thiamine deficiency in pigs. Am. J. Path. (In press.)

MACKENZIE, C. G., M. D. LEVINE AND E. V. McCOLLUM 1940 The prevention and cure of nutritional muscular dystrophy in the rabbit by alpha-tocopherol in the absence of a water-soluble factor. J. Nutrition, vol. 20, p. 399.

PENNINGTON, D., E. E. SNELL AND R. J. WILLIAMS 1940 An assay method for pantothenic acid. J. Biol. Chem., vol. 135, p. 213.

SCUDI, J. V. 1941 On the colorimetric determination of vitamin $B_6$. J. Biol. Chem., vol. 139, p. 707.

STRONG, F. M., R. E. FEENEY, B. MOORE AND H. T. PARSONS 1941 The riboflavin content of blood and urine. J. Biol. Chem., vol. 137, p. 363.

SWANK, R. L. 1940 Avian thiamin deficiency. A correlation of the pathology and clinical behavior. J. Exp. Med., vol. 71, p. 683.

SWANK, R. L., R. R. PORTER AND A. YEOMANS 1941 The production and study of cardiac failure in thiamin-deficient dogs. Am. Heart J., vol. 22, p. 154.

WINTROBE, M. M., R. H. FOLLIS, JR., M. H. MILLER, H. J. STEIN, S. HUMPHREYS AND A. SUKSTA 1942 Pyridoxine deficiency in swine with special reference to anemia, epileptiform convulsions and fatty liver. To be published.

WINTROBE, M. M., J. L. MILLER, JR. AND H. LISCO 1940 The relation of diet to the occurrence of ataxia and degeneration in the nervous system of pigs. Bull. Johns Hopkins Hosp., vol. 67, p. 377.

WINTROBE, M. M., D. M. MITCHELL AND L. C. KOLB 1938 Sensory neuron degeneration in vitamin deficiency. Degeneration of the posterior columns of the spinal cord, peripheral nerves, and dorsal root ganglion cells in young pigs fed a diet containing thiamin ($B_1$) and riboflavin but otherwise deficient in vitamin B complex. J. Exp. Med., vol. 68, p. 207.

WINTROBE, M. M., C. MUSHATT, J. L. MILLER, JR., L. C. KOLB, H. J. STEIN AND H. LISCO 1942 The prevention of sensory neuron degeneration in the pig, with special reference to the role of various liver fractions. J. Clin. Invest., vol. 21, p. 71.

WINTROBE, M. M., H. J. STEIN, M. H. MILLER, R. H. FOLLIS, JR., V. NAJJAR AND S. HUMPHREYS 1942 A study of thiamine deficiency in swine. Together with a comparison of methods of assay. Bull. Johns Hopkins Hosp., vol. 71, p. 141.

# CALCIUM, PHOSPHORUS AND NITROGEN METABOLISM OF YOUNG COLLEGE WOMEN [1]

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THREE FIGURES

Although the years of late adolescence and early adulthood of women are important from the standpoint of nutrition, little is known concerning the nutrients ingested or the retention of such nutrients by young women in this age group. Even a casual observation of the food eaten by a group of individuals shows wide differences in food habits; differences which are largely due to custom, which in turn has been influenced by many factors, including the economic one. The controlled diets of balance experiments are duplicated to a very small extent by the population at large. Outhouse et al. ('41) showed that calcium needs, computed for individuals on fixed diets, could apply only to individuals who were on similar diets.

Information as to the amounts of nutrients ingested and retained when women are eating their customary diets should

undoubtedly be considered in any discussion of recommended food allowances. A few studies have been made on intakes and retentions of women on their self-chosen diets (Wang, Hawks, Huddlestun, Wood and Smith, '30; Hetler, '32; Coons and Schiefelbusch, '32; Hunscher, Donelson, Erickson and Macy, '34).

As part of a long-time study of the nutritional status of college women as related to their dietary habits (Nelson, '38), a study of calcium, phosphorus and nitrogen intakes and retentions or losses was made of a large group of young college women during the years of 1936 to 1941. The results of these studies which are here reported are from three sources: Part I, which dealt with a group of college women observed during one or more 7- or 10-day periods during which the women ate their customary self-chosen diets; Part II, which dealt with one woman studied for forty-four 5-day periods while she was on her usual diet; and Part III, which dealt with a group of nine women studied for three periods of 10 days each while the women were eating basal diets supplemented by definite amounts of milk. Most of the observations were made between menstrual periods.

The subjects were all presumably in good health, carrying on their usual curricular and extracurricular activities, and continued their usual manner of life during the period of the study. Living and eating arrangements of the women considered in Parts I and II were representative of living arrangements of young women enrolled in land-grant colleges. The group included young women who had their meals at home; at sorority houses; at university houses, where the women prepared their own meals; at university eating places; and at university residence halls. Whatever the living arrangement, some choices as to foods eaten were inevitable, and foods eaten during the time each girl's balance was studied represented her customary diet.

Of a total of 109 individuals included in Part I, fourteen were observed at intervals during 2 or more years. For example, one girl's balance was studied when she was 19, again

when she was 20, and then again when she was 21, age being calculated to the nearest birthday. In order to study any effect which age might have on retention, it was decided to include each of these subjects in each of the age groups in which she was at the time of the observation. Considering an individual in each age group in which she was at the time of observation, the total number of observations are reported as 124 rather than 109. Of the 124 observations, eighty-four were made in Nebraska, twenty-eight in Ohio, nine in Kansas, and three in Iowa.

Daily records of foods with amounts eaten were kept for every subject during each observation period. Inspection of the records showed that the majority of the subjects used some foods from each of the following groups during each period: milk and milk products; potatoes and sweet potatoes; tomatoes and citrus fruits; leafy, green, and yellow vegetables; other fruits and vegetables; eggs; lean meat, poultry or fish; flour and cereals; fat including butter; and sugar. Amounts of the different foods used varied and the quality of each subject's diet depended upon the amounts used from each food group. Many of the diets were undoubtedly above the average in quality.

Because it was found early in the study that A. H. in Iowa, the individual included in Part II, failed to retain calcium on an intake of less than 1 gm., her daily food intake was planned to allow 4 cups of milk. Otherwise her diet was unrestricted.

During the school years 1939–1940 and 1940–1941, a study was made of the calcium, phosphorus, and nitrogen intakes and retentions of nine young women when they were on basal diets supplemented during each of three periods by 1, 2, and 3 cups of milk, respectively. The basal diets used were adaptations of diets used in a metabolism study by Leverton and Roberts ('37). Foods included in the basal diets during each period were the same in kind and amount for each individual and were such that, with the exception of calcium, the diets as a whole were adequate. The amounts of calcium in the diets

varied with the amounts of milk used. Certain foods of negligible mineral content, such as white bread, butter, jelly, sugar, and candy, were used ad libitum to meet the varying calorie needs of the individuals.

These young women lived in their usual places of residence. Their meals, planned to include the basal diet plus the designated amounts of milk, were prepared and served to them.

This study was divided into a 10-day collection period at each level of milk intake, each collection period being preceded by a 15-day foreperiod. Studies of four of the nine subjects on the controlled diets were made in Ohio, three in Iowa, and two in Kansas.

## METHODS

The customary procedure used in collecting food samples and excreta was used. Food was weighed as eaten and sampled, and samples were pooled into a composite representing an aliquot of each day's intake for each individual. At the end of the period each composite sample was digested with concentrated HCl, sieved, made up to a convenient volume, thoroughly mixed and stored for analysis at a later date. Total feces for the individual for each period were collected, carmine being used as a marker, and the composite sample was made into an acid digest and stored. Urine was collected daily, aliquots were acidified with HCl, and the composite of the aliquots for each subject was analyzed, without preliminary ashing. Aliquots of the acid digests of food and feces were ashed for calcium and phosphorus determinations according to the method of Stearns ('29). For calcium determinations a modification of the McCrudden method as described by Stearns ('29) was used; for phosphorus, the Fiske and Subbarow method ('25); and for the nitrogen, the Kjeldahl procedure, (Association of Official Agricultural Chemists, '30). Similar procedures in collection and preparation of samples and in chemical analysis were followed in each of the four states.

### RESULTS AND DISCUSSION

For the 124 women included in Part I of the study, certain decisions to be made concerning the validity of using the data so collected necessitated statistical analysis. The method of variance and covariance as described by Snedecor ('40) was used as an aid in making these decisions as indicated in the following paragraphs.

### Intra-individual differences

In some cases a young woman's balance was studied for two or more periods during 1 year. Variations from period to period of such individuals were significantly less than variations from individual to individual. As shown by the mean squares of retention adjusted for intake, the intra-individual variance was 0.011 as compared to an inter-individual variance of 0.024. Accordingly the averages for individuals observed more than once during a year have been reported.

### Place differences

Mean intakes and retentions differed from state to state. Analysis of the data showed that variance in retentions could be reduced by adjusting to the mean intake. The difference between the retentions adjusted for intake of the Nebraska and the Ohio groups was found not to be significant, as indicated by the use of Student's t-test. The number of cases in the other two groups were too small to justify analysis or to have much influence on the figures for the entire group. Consequently state differences have been disregarded in the study of the group of 124 individuals.

### Age differences

Jeans and Stearns ('39) quote Todd as stating that mineralization of the skeleton does not always keep pace with the rate of skeletal growth. For some time after growth ceases, one might expect a continued deposition of skeletal minerals, and this deposition would be reflected in the greater mineral

retention during such periods if adequate intakes were the rule. The exact age at which complete mineralization occurs probably varies widely among individuals. Analysis of the data by age groups for these young college women, however, showed mean calcium retentions which did not differ significantly among the various age groups. Possibly physiological and chronological ages may have varied to such an extent in each age group that the two were confused. Certainly no indication as to when storage ceased was shown by the data. A detail analysis, using age in years and fractions thereof, showed no relation between balance and age for the one subject, A.H. Age differences have therefore also been disregarded in the results reported.

### Weight differences

An analysis, by means of multiple regression of calcium intake and body weight upon calcium retention, gave a correlation coefficient, $R = 0.504$, which was only slightly greater than the correlation coefficient of calcium intake upon calcium retention, $r = 0.502$. In addition, calcium intake was not significantly related to weight as shown by an r of 0.078. Weight differences have therefore been disregarded in the results presented.

The data for the 124 women as reported therefore, include mean daily figures for each individual even though some individuals were studied more than once during a year, and disregard place, age, and weight differences.

### Calcium intakes

The mean daily calcium intake, 0.941 gm. (table 1) for the large group of 124 women was similar to the daily mean intakes of 0.93 and 0.95 gm. reported by Coons and Schiefelbusch ('32) and Hunscher et al. ('34), respectively, and came within the range of what Sherman and Lanford ('41) refer to as a desirable population standard of from 0.9 to 1 gm. per capita daily. The mean daily intake for the large Nebraska

## TABLE 1

### Calcium, phosphorus and nitrogen metabolism data according to intake levels for 195 observations (in grams).

| | CUSTOMARY SELF-CHOSEN DIETS | | | | | | | | CONTROLLED DIETS | | | |
| | Part I. 124 individuals with 124 studies | | | | Part II. One individual (A.H.) with 44 studies | | | | Part III. Nine individuals with 27 studies | | | |
| DAILY INTAKE RANGE | No. of studies | Mean intake | Mean balance | Percentage Individuals Retaining | No. of studies | Mean intake | Mean balance | Percentage Individuals Retaining | No. of studies | Mean intake | Mean balance | Percentage Individuals Retaining |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Calcium** | | | | | | | | | | | | |
| 0.322–.399 | 1 | 0.322 | —.089 | 0 | .. | .... | ... | .. | .. | .... | ... | ... |
| 0.400–.499 | 6 | 0.469 | —.139 | 17 | .. | .... | ... | .. | .. | .... | ... | ... |
| 0.500–.599 | 11 | 0.561 | —.025 | 36 | .. | .... | ... | .. | 3 | 0.560 | .019 | 100 |
| 0.600–.699 | 14 | 0.659 | —.033 | 43 | .. | .... | ... | .. | 5 | 0.648 | .035 | 60 |
| 0.700–.799 | 12 | 0.753 | —.040 | 50 | .. | .... | ... | .. | 2 | 0.765 | .028 | 50 |
| 0.800–.899 | 16 | 0.850 | .034 | 62 | .. | .... | ... | .. | 3 | 0.809 | —.061 | 0 |
| 0.900–.999 | 13 | 0.939 | —.019 | 46 | 7 | 0.934 | —.110 | 0 | 2 | 0.940 | .067 | 100 |
| 1.000–1.099 | 18 | 1.045 | .044 | 72 | 5 | 1.058 | .042 | 80 | 3 | 1.061 | .133 | 100 |
| 1.100–1.199 | 12 | 1.145 | .111 | 83 | 2 | 1.108 | .074 | 100 | 5 | 1.147 | .104 | 100 |
| 1.200–1.299 | 4 | 1.261 | .125 | 100 | 13 | 1.255 | .043 | 62 | 3 | 1.264 | .076 | 100 |
| 1.300–1.399 | 6 | 1.344 | .142 | 83 | 9 | 1.359 | .180 | 89 | 1 | 1.398 | .100 | 100 |
| 1.400–1.499 | 4 | 1.464 | .227 | 100 | 6 | 1.429 | .108 | 83 | .. | .... | ... | ... |
| 1.500–1.599 | 4 | 1.523 | .234 | 75 | 1 | 1.552 | .362 | 100 | .. | .... | ... | ... |
| 1.600–2.323 | 3 | 1.885 | .180 | 100 | 1 | 1.653 | .309 | 100 | .. | .... | ... | ... |
| Means | | 0.941 | .030 | | | 1.236 | .072 | | | 0.921 | .055 | |
| **Phosphorus** | | | | | | | | | | | | |
| 0.663–.699 | 3 | 0.675 | —.136 | 0 | .. | .... | ... | .. | .. | .... | ... | ... |
| 0.700–.799 | 10 | 0.762 | —.140 | 10 | .. | .... | ... | .. | .. | .... | ... | ... |
| 0.800–.899 | 11 | 0.853 | —.049 | 36 | .. | .... | ... | .. | .. | .... | ... | ... |
| 0.900–.999 | 7 | 0.951 | .062 | 86 | 1 | 0.990 | —.177 | 0 | 4 | 0.956 | .100 | 100 |
| 1.000–1.099 | 20 | 1.052 | —.042 | 40 | 5 | 1.026 | .016 | 60 | 2 | 1.060 | .146 | 100 |
| 1.100–1.199 | 21 | 1.145 | .109 | 90 | 7 | 1.151 | .145 | 86 | 7 | 1.141 | .002 | 57 |
| 1.200–1.299 | 18 | 1.246 | .103 | 89 | 15 | 1.242 | .168 | 93 | 2 | 1.277 | .116 | 100 |
| 1.300–1.399 | 7 | 1.351 | .175 | 100 | 9 | 1.357 | .181 | 89 | 6 | 1.355 | .054 | 50 |
| 1.400–1.499 | 9 | 1.432 | .144 | 89 | 5 | 1.424 | .302 | 100 | 2 | 1.441 | .161 | 100 |
| 1.500–1.599 | 7 | 1.544 | .161 | 100 | 2 | 1.542 | .290 | 100 | 2 | 1.592 | .291 | 100 |
| 1.600–1.699 | 3 | 1.668 | .197 | 100 | .. | .... | ... | .. | 2 | 1.673 | .284 | 100 |
| 1.700–1.799 | 4 | 1.742 | .331 | 100 | .. | .... | ... | .. | .. | .... | ... | ... |
| 1.800–2.125 | 4 | 1.901 | .185 | 100 | .. | .... | ... | .. | .. | .... | ... | ... |
| Means | | 1.177 | .062 | | | 1.255 | .164 | | | 1.260 | .101 | |
| **Nitrogen** | | | | | | | | | | | | |
| 5.55–5.99 | 1 | 5.55 | —1.67 | 0 | .. | .... | ... | .. | .. | .... | ... | ... |
| 6.00–6.99 | 3 | 6.70 | —0.72 | 33 | .. | .... | ... | .. | .. | .... | ... | ... |
| 7.00–7.99 | 14 | 7.51 | —0.62 | 14 | .. | .... | ... | .. | .. | .... | ... | ... |
| 8.00–8.99 | 19 | 8.51 | 0.02 | 63 | 4 | 8.78 | 0.26 | 50 | 2 | 8.85 | 0.70 | 100 |
| 9.00–9.99 | 26 | 9.52 | 0.38 | 69 | 17 | 9.60 | 1.22 | 100 | 2 | 9.06 | 0.19 | 50 |
| 10.00–10.99 | 26 | 10.45 | 1.09 | 92 | 15 | 10.43 | 1.71 | 100 | 7 | 10.45 | 0.64 | 86 |
| 11.00–11.99 | 15 | 11.47 | 1.07 | 80 | 6 | 11.11 | 2.79 | 100 | 6 | 11.36 | 0.86 | 83 |
| 12.00–12.99 | 11 | 12.38 | 1.37 | 100 | 2 | 12.28 | 3.05 | 100 | 7 | 12.27 | 1.36 | 86 |
| 13.00–13.99 | 3 | 13.41 | 1.53 | 100 | .. | .... | ... | .. | 3 | 13.51 | 1.20 | 100 |
| 14.00–14.99 | 3 | 14.65 | 2.59 | 100 | .. | .... | ... | .. | .. | .... | ... | ... |
| 15.00–17.58 | 3 | 16.27 | 2.89 | 100 | .. | .... | ... | .. | .. | .... | ... | ... |
| Means | | 10.10 | 0.63 | | | 10.13 | 1.59 | | | 11.24 | 0.91 | |

group (0.856 gm.) was practically the same as the slightly lower daily allowance for women recommended by the Committee on Food and Nutrition of the National Research Council ('41).

Variations in intake among individual subjects were large and ranged from a low of 0.322 gm. (B.B. in Ohio) to a high of 2.323 gm. (R.H. in Kansas), but observation of foods used by groups of individuals shows that calcium intakes do vary widely and that the variation as shown by this group of young women probably presents a typical picture of the custom of the general population of young college women in regard to calcium intake (fig. 1).
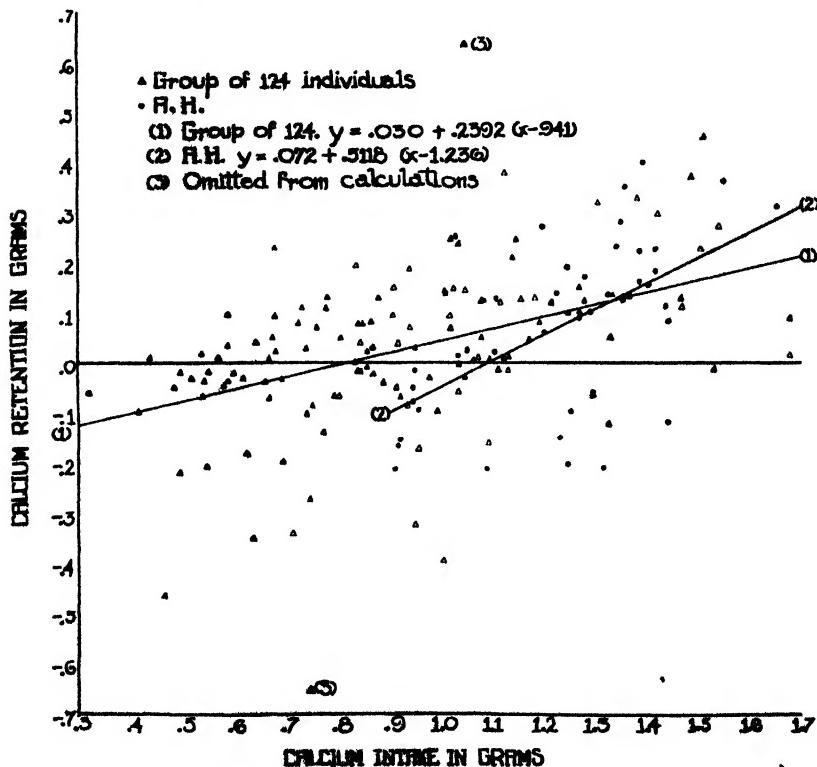


Fig. 1   The regression of calcium retention on calcium intake. One individual, intake 2.323 gm. and retention 0.217 gm., was omitted from the graph, but the figures were included in the calculation of the regression coefficient.

As shown in table 1, forty-four women or 35% of the 124, had less than the recommended 0.8 gm. daily.

The individual, A.H., for whom results are considered as of Part II, had a generous calcium intake, which ranged from a daily mean of 0.910 to 1.653 gm. with a mean of 1.236 for the entire forty-four studies. This amount exceeded the mean of the group of 124 individuals by 0.295 gm.

Calcium intakes of the nine women considered in Part III varied with the level of milk used, mean daily figures for the group being 0.629, 0.921, and 1.214 gm. as 1, 2 or 3 cups of milk, respectively, were used in addition to the basal diet. At the 1-cup level of milk ingestion the calcium intakes were all less than the recommended 0.8 gm. daily.

### Calcium retentions

For the 124 women, retention was significantly related to intake as shown by a coefficient of correlation of 0.50. Losses were more frequent than were retentions at mean intake levels of less than 0.8 gm., with the percentage of individuals who were storing calcium at these low levels increasing from 0 at an intake of 0.3 gm. to 50 at an intake of 0.7 to 0.8 gm. (table 1). At intake levels of 0.8 gm. and above, mean retentions were evident at all but one level (0.900–0.999), with the percentage of individuals storing calcium increasing fairly consistently as the intake level increased.

Mean daily retentions varied among individuals on similar intakes. For example, twelve individuals having mean daily intakes ranging from 1.006 to 1.048 had balances ranging from —0.394 to 0.250 (fig. 1).

It is a commonly-accepted fact that even on a controlled diet an individual's retention may vary from period to period; and that different individuals show varying responses to similar intakes. Steggerda and Mitchell ('41) and Breiter et al. ('41) report extreme variability of calcium retentions for adults on the same types of diet. The variations in retentions found among these individuals on similar calcium

intakes in which the diets varied in other factors, are therefore not surprising.

Reasons for differences in response are obscure. It has been assumed that well-nourished individuals utilize calcium less efficiently than those whose skeletal tissues have been depleted, but Breiter et al. ('41) found that of seven subjects observed, the three who showed the highest utilization of calcium were well-nourished in regard to this element.

The regression of balance on intake (fig. 1) shows the rate at which retention may be expected to increase when intake is increased as calculated from the data obtained for the 124 women. The regression line intersects the line of equilibrium at the point 0.816 gm. The indication therefore is that the young women in this group of 124 required an intake of that amount (0.816 gm.) to maintain themselves in equilibrium while they were eating their customary diets.

For A.H. (Part II), also, retention was significantly dependent upon intake, as shown by a coefficient of correlation of 0.56.

No retentions were evident at an intake level of less than 1 gm. Average figures showed retention at each intake level of 1 gm. and over, but the percentage of retentions at each level fluctuated to a greater extent than was the case for the group reported in Part I.

The regression of balance on intake for A.H. (fig. 1) shows that although her retention increased with increased intake the regression line intersects the line of equilibrium at 1.095 as compared to 0.816 for the large group.

When the mean daily calcium intakes of the nine women (Part III) ranged from 0.5 to 0.8 gm. the mean daily retentions were 0.019, 0.035, and 0.028 (table 1) for the three intake levels represented. Corresponding figures for the group of 124 (Part I) were —0.025, —0.033, and —0.040. The differences between the response of the two groups at the three levels were 0.044, 0.068 and 0.068 gm., respectively. Although these differences were small, they made storage possible. In addition, 70% of the group on the controlled diets

at these levels stored calcium at intake levels of less than 0.8 gm. as compared to 43% of the group who were eating their customary diets.

At intake levels above 0.8 gm., differences between retentions of the women on the controlled and those on their customary diets were less pronounced, the mean daily retention of the former being 0.070 gm. as compared to a corresponding figure of 0.055 gm. for the latter. Eighty-two per cent of the women on controlled diets stored calcium, as compared to 70% of those on their usual diets.

Undoubtedly the amount of calcium in the self-chosen diets was directly related to the milk intakes. Increased amounts of milk improve the quality of the diet in other factors as well as in calcium. At the lower levels of calcium intake, a decidedly larger proportion of the group having the controlled diets were in equilibrium or were storing calcium than was the case with the group who were having their customary self-chosen diets. It seems logical to assume that the smaller amounts of calcium were therefore more efficiently utilized when the diet was so selected as to provide adequately for other dietary factors than was the case when the women were on their customary diets.

## Phosphorus intakes

The mean daily phosphorus intake for the group of 124 (1.177 gm.) (table 1) was similar to the 1.19 gm. reported by Coons and Schiefelbusch ('32), lower than the 1.523 gm. reported by Hunscher et al. ('34) and lower than the commonly-accepted Sherman standard of 1.32 gm. Inspection of table 1 shows that although the range in mean daily phosphorus intakes was large (0.663–2.125) the intakes clustered around the mean to a larger extent than was the case in regard to calcium. This was shown by a coefficient of variation of 25% for phosphorus as compared to a coefficient of variation of 34% for calcium.

Mean phosphorus intake for A.H. (Part II) was 1.255 with a range of from 0.990 to 1.574 and a coefficient of variation

of 11%, as compared to a coefficient of variation of 15% for calcium intake. Her mean intake for the forty-four periods of study (1.255) was only slightly below the Sherman standard.

Mean daily intakes of the group of nine women on controlled diets increased as the level of milk increased, mean daily intake figures for the group being 1.044, 1.245 and 1.492 gm. as 1, 2 or 3 cups of milk were used in addition to the basal diet.

### Phosphorus retentions

For the group of 124 women, the phosphorus retentions were significantly dependent upon intake, as shown by a coefficient of correlation of 0.58. Losses were more frequent than retentions at intake levels of less than 1.1 gm. At levels of 1.1 and above, 93% of the individuals were retaining phosphorus. Figure 2 shows the regression of balance on



▲ Group of 124 individuals
● R.H.
(1) Group of 124. y = .062 + .3694 (x-1.177)
(2) R.H. y = .164 + .5719 (x-1.255)
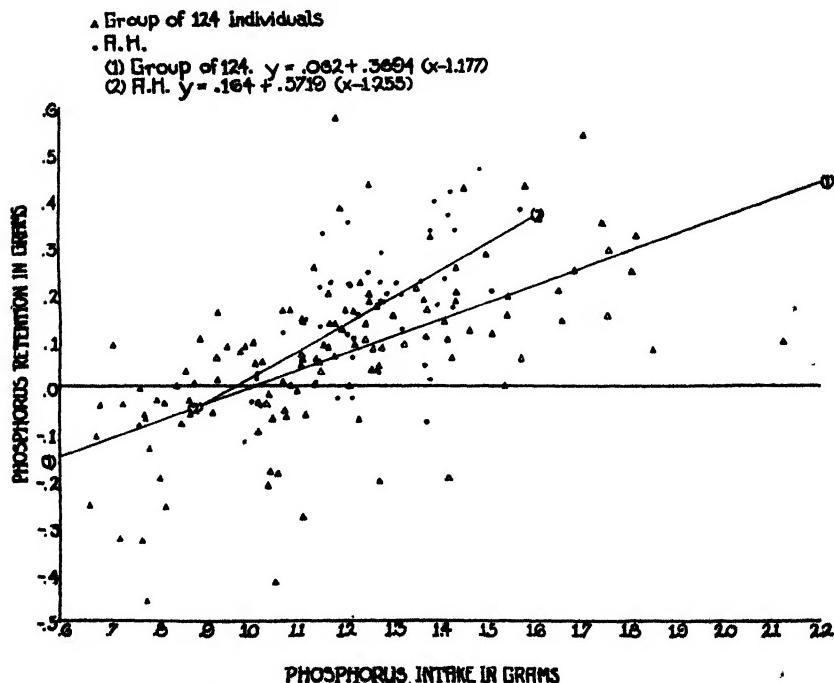
Fig. 2 The regression of phosphorus retention on phosphorus intake.

intake, and indicated that, for this group, an intake of about 1 gm. was necessary for the women to maintain themselves in equilibrium while they were eating their customary diets.

For A.H. (Part II) phosphorus retentions were significantly dependent upon intake, as shown by a coefficient of correlation of 0.59. As with the large group, losses were more frequent at intake levels of less than 1.1 gm., than at the higher levels, the percentages of retentions increasing fairly consistently as intakes increased.

The regression of balance on intake for A.H. (fig. 2) indicates that an intake of about 1 gm. was necessary for her to maintain herself in equilibrium.

Mean daily phosphorus retentions of the group of nine women on the basic diet supplemented by 1, 2, and 3 cups of milk, respectively, did not increase with regularity as intakes increased. However, the amount retained was greatest at the 3-cup level. At intakes of less than 1.1 gm., mean retentions were larger and the percentage of retentions greater than was the case for the 124 individuals and for A.H. on their usual diets. At intakes of 1.1 gm. and above, no evidence of better retention by the group on the controlled diet was shown by the figures.

### Nitrogen intakes

Mean daily intakes of the group of 124 ranged from 5.55 gm. to 17.58 gm. with a mean of 10.10 gm. which varied only slightly from the daily allowance of 60 gm. of protein recommended by the Committee on Food and Nutrition of the National Research Council (table 1).

The coefficient of variation (20%) was less than that for calcium or phosphorus and indicated less variability in nitrogen intake than was the case for either calcium or phosphorus.

For A.H. (Part II) the mean daily intake of 10.13 gm. was almost identical with that of the large group. A coefficient of variation of 8% indicated less variability from the mean than was the case for either calcium or phosphorus.

Mean daily nitrogen intakes of the group of nine women eating the controlled diets increased consistently as the level of milk intake increased, and ranged from 8.85 gm. to 13.51 gm.

### Nitrogen retentions

Retention of nitrogen for the group of 124 individuals was significantly related to intake as shown by a coefficient of
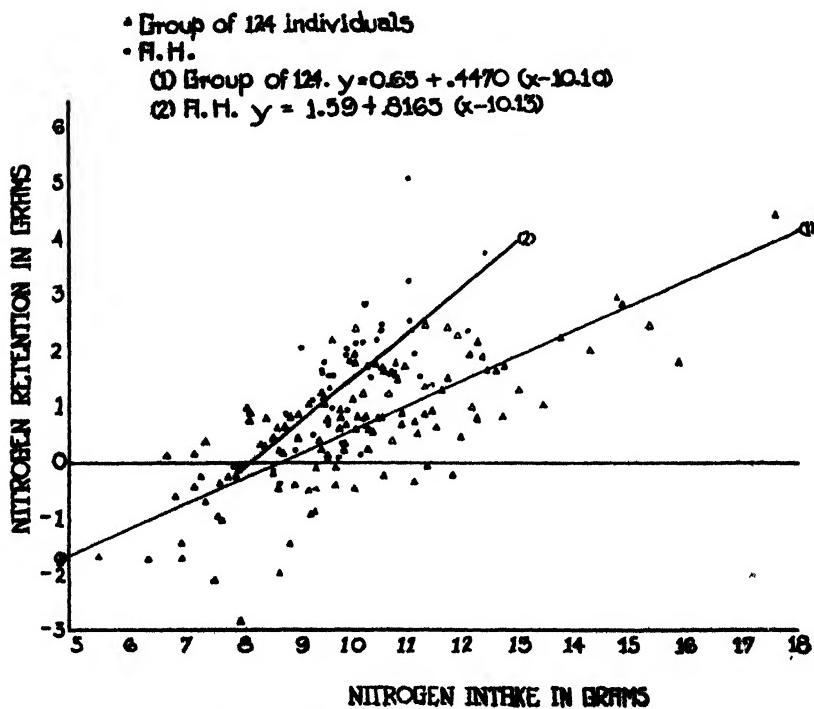


Fig. 3   The regression of nitrogen retention on nitrogen intake.

correlation of 0.73. At the lower levels of intake (less than 8 gm.) few retentions were observed. At intakes of 8 gm. and above, mean daily retentions as well as the percentage of the group storing nitrogen increased fairly consistently as intakes increased. Of the twenty individuals who had a mean intake of 12 gm. or more daily, all were storing nitrogen.

The regression of nitrogen balance on intake for the group of 124 indicated that at an intake of 8.69 gm., equilibrium might be expected for the group.

At mean daily intake levels of 9 gm. and above, A.H. stored nitrogen, the amounts stored increasing as intake increased. Regression of balance on intake showed that the intakes necessary for equilibrium for both the group of 124 and A.H. were similar.

Mean daily retentions for the nine women on the controlled diets increased slightly but consistently as milk intakes increased, but showed no evidence of better utilization of nitrogen than did the retentions for those women who were using their customary self-chosen diets.

### Relative intakes and retentions of calcium, phosphorus and nitrogen

For the group of 124 women, coefficients of correlation of intake on retention were 0.50, 0.58 and 0.73 for calcium, phosphorus and nitrogen, respectively, and showed that phosphorus and nitrogen retentions were more closely dependent upon intake than was the case in regard to calcium. For A.H. (Part II) the coefficients of correlation for calcium and phosphorus intake on retention did not differ greatly, being 0.56 and 0.59, respectively, with a corresponding coefficient of correlation of 0.66 for nitrogen. A larger proportion of calcium than of either phosphorus or nitrogen is excreted in the feces. Because the marking of the feces is difficult, errors from this source tend to contribute to greater variation in calcium figures than in either phosphorus or nitrogen figures. This may have a bearing on the fact that retention of calcium, in contrast to the other two elements, appeared to be more dependent upon factors other than intake. This same source of error would operate similarly in all the studies.

At mean daily calcium intake levels ranging from 0.5 to 1.4 gm. there were (a) 106 studies of women on their self-chosen diets, (b) thirty-six studies for A.H., and (c) twenty-seven

studies for the nine women on the controlled diets. Of these, retentions were found for (a) 60% of the 106 studies of the women on their usual diets, (b) 61% of the A.H. studies, and (c) 78% of the studies of women on the controlled diets. These differences seem large enough to warrant the conclusion that the nature of the diet was a factor in calcium retentions and that the controlled diets had a stabilizing effect upon such retentions.

Milk, the food most logically used to increase the calcium intake, improves the diet also in factors other than calcium and tends, therefore, to improve calcium retention. Many young college women have diets which are far from adequate and undoubtedly this is also the case in regard to other young women. The desirability of a generous allowance of calcium for young women in general seems indicated by the findings of this study.

### Phosphorus retention as related to calcium and nitrogen retentions.

Because the metabolism of phosphorus is related to that of nitrogen as well as of calcium, interrelationships of the three elements are of interest. It is usually assumed that in the adult body, 1 part of phosphorus combines with 17 parts of nitrogen and 2.18 parts of calcium, giving rise to the retention formula $P = \frac{Ca}{2.18} + \frac{N}{17}$ (Stearns, '33). By means of this formula, the phosphorus retentions were computed for (a) the group of 124 women, (b) A.H., and (c) the group of nine women. Computed retentions as compared to actual retentions were, respectively, as follows: (a) 0.051 and 0.062, (b) 0.127 and 0.164, and (c) 0.079 and 0.101.

These results of comparing actual and computed phosphorus retentions may indicate that a study of intakes and retentions of a large group of individuals, even though the periods of study are short, may show a better picture of the needs of individuals of corresponding ages than a more intensive study of a smaller group of individuals or of one individual.

Individual differences are minimized in the former case, whereas in the latter cases they tend to influence the results.

### SUMMARY AND CONCLUSIONS

Calcium, phosphorus and nitrogen intakes and retentions were studied for (a) 124 college women from four states, on their customary self-chosen diets; (b) an individual for forty-four 5-day periods at intervals during her nineteenth through her twenty-second year; and (c) a group of nine young women eating basal diets supplemented with 1, 2, and 3 cups of milk, respectively. Results showed that place, age and weight differences were not significant for the 124 subjects, and that intake was significantly related to retention. Figures of regression of balance upon intake showed that mean daily intakes of 0.8 gm. calcium, 1 gm. phosphorus and 9 gm. nitrogen, respectively, were required for equilibrium. Corresponding figures for the one subject were 1.1, 1, and 8 gm.

That the type of diet was one of the factors influencing calcium retention was shown by the larger percentage of calcium retentions of subjects on controlled diets as compared with those on their customary self-chosen diets, when the calcium intakes were similar.

In view of the fact that diets probably vary among the general population to a greater extent than did those used by the group of college women studied, the desirability of a generous calcium allowance for young women of college age (17 to 24) seems indicated, and the calcium allowance of 1 gm. recommended by the Committee on Food and Nutrition of the National Research Council for girls of 16 to 20 might well be recommended for the group 17 to 24 years old.

For the large group of 124, calcium, phosphorus and nitrogen retention relationship was similar to the relationship commonly accepted. This was not the case for the individual whose balances were studied intensively. For the purposes of estimating population needs, it would seem that a short-time study of a large group is more desirable than the intensive study of one individual.

## LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1930 Official and tentative methods of analysis. The Association, Washington, D. C., p. 18.

BREITER, H., R. MILLS, J. DWIGHT, B. McKEY, W. ARMSTRONG AND J. OUTHOUSE 1941 The utilization of the calcium of milk by adults. J. Nutrition, vol. 21, p. 351.

COMMITTEE ON FOOD AND NUTRITION, NATIONAL RESEARCH COUNCIL 1941 Recommended allowances for the various dietary essentials. J. Am. Diet. Assoc., vol. 17, p. 565.

COONS, C. M., AND A. T. SCHIEFELBUSCH 1932 The diets of college women in relation to their basal metabolism. J. Nutrition, vol. 5, p. 459.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

HETLER, R. A. 1932 Protein intake and basal metabolism of college women. J. Nutrition, vol. 5, p. 69.

HUNSCHER, H. A., E. DONELSON, B. N. ERICKSON AND I. G. MACY 1934 Results of the ingestion of cod liver oil and yeast on calcium and phosphorus metabolism of women. J. Nutrition, vol. 8, p. 341.

JEANS, P. C., AND G. STEARNS 1939 The human requirement of vitamin D. The vitamins. A symposium. The American Medical Association, Chap. 26, p. 485.

LEVERTON, R. M., AND L. J. ROBERTS 1937 The iron metabolism of normal young women during consecutive menstrual cycles. J. Nutrition, vol. 13, p. 65.

NELSON, P. M. 1938 The nutritional status of college women. J. Home Econ., vol. 30, p. 465.

OUTHOUSE, J., H. BREITER, E. RUTHERFORD, J. DWIGHT, R. MILLS AND W. ARMSTRONG 1941 The calcium requirement of man: balance studies on seven adults. J. Nutrition, vol. 21, p. 565.

SHERMAN, H. C., AND C. S. LANFORD 1941 The Essentials of Nutrition, Macmillan Company, New York, p. 135.

SNEDECOR, G. W. 1940 Statistical methods applied to experiments in agriculture and biology. Third ed. The Iowa State College Press, Ames, Iowa.

STEARNS, G. 1929 A rapid method for the preparation of fecal digests suitable for use in nitrogen and mineral analyses. J. Lab. and Clin. Med., vol. 14, p. 954.

———— 1933 Soybean flour in infant feeding. Amer. J. Dis. Child, vol. 46, p. 7.

STEGGERDA, F. R., AND H. H. MITCHELL 1941 Further experiments on the calcium requirement of adult man and the utilization of the calcium in milk. J. Nutrition, vol. 21, p. 577.

WANG, CHI CHE, J. E. HAWKS, B. HUDDLESTUN, A. A. WOOD AND E. A. SMITH 1930 The influence of high and low protein diets on the basal metabolism and the chemistry of blood and urine in normal women. J. Nutrition, vol. 3, p. 79.

# SOYBEAN PROTEIN AS A SOURCE OF AMINO ACIDS
# FOR THE CHICK

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One of the most interesting characteristics of the soybean
protein is the great increase in its nutritional value after
sufficient heat treatment. The nature of the difference be-
tween raw and heated soybean protein has been shown to
involve the availability of the sulphur-bearing amino acids.
The literature on this topic has been thoroughly reviewed by
Hayward and Hafner ('41). These authors concluded, on
the basis of experiments with both chicks and rats, that their
diets containing raw soybean protein were deficient in avail-
able cystine and supplied a positive but sub-optimal level of '
available methionine.

In all of the studies reported so far, the chick diets have
contained appreciable amounts of protein other than that of
the soybean. It is, therefore, not possible to attribute the
results with these diets entirely to the soybean protein. An
effort to avoid this difficulty was made by the use of a purified
diet which contained no form of protein other than that
furnished by the soybean or soybean meal. At the same time
the now well-proven multiple function of methionine was
given consideration. Methionine in the diet is the sole source
of methionine in the animal tissues, but it may also furnish
the sulphur for the synthesis of cystine and the methyl groups
for the synthesis of choline and creatine. The methionine
requirement for growth may, therefore, be affected by the
presence or absence of dietary choline and cystine in so far

as these compounds in the diet may influence their rate of formation from methionine in vivo.

Studies with a few other amino acids were conducted to obtain further information on the completeness of heated soybean protein for the chick.

### METHODS AND RESULTS

The basal diet employed in this work contained the following per 100 gm.: calcium gluconate 5.0, ox-bile salts 0.25, fish oil (400D–5000A) 0.25, tricalcium phosphate 2.0, dipotassium phosphate 0.5, potassium chloride 0.5, potassium iodide 0.001, sodium chloride 0.5, sodium silicate 0.2, manganese sulphate monohydrate 0.1, and magnesium oxide 0.2 gm. Water-soluble vitamins were added in the form of thiamine 1, riboflavin 1, pyridoxine 1, nicotinic acid 0.5 and calcium pantothenate 3 mg. An extract of brewer's yeast, prepared by leaching with 50% methanol and concentrating under reduced pressure, was added at a level equivalent to 5 gm. of yeast.

The variables in the diets were ground raw soybeans, commercial solvent-process heated soybean meal, pure amino acids, gelatin, soybean oil and glucose. The different forms of soybean protein were added to the diets in amounts to provide 20 gm. of crude protein per 100 gm. of diet as determined by analysis. Gelatin and amino acids, where added, were in addition to the soybean protein. Crude soybean oil in the amount of 5 gm. was added with the heated soybean meal. Finally, glucose was added to complete the 100 gm.

The chicks were Single Comb White Leghorn stock that had been reared for 1 week on a practical diet, then carefully selected for uniform weight and vigor and placed in experimental groups of eight chicks each. The chicks were housed in all-metal, electrically-heated battery brooders and given the diets and water ad libitum. Weighings were made at intervals of 3 to 4 days. The experimental period lasted from 10 to 14 days. The course of an experiment was almost invariably clearly indicated in the first few days. This has been generally true of chick amino acid studies.

The growth results are expressed in the form of average percentage gain per day. This is the average gain divided by the average weight of the chicks during the experiment and the time in days, and multiplied by 100. For the strain of chicks and the age period used, this percentage gain is close to 7.0 when the chicks are fed a practical rearing diet with approximately 20% crude protein.

The methionine content of the raw soybean protein was 1.9% and that of the soybean meal protein 2.1%. The gelatin used contained less than 0.1% methionine. The determinations of methionine were made by the method of McCarthy and Sullivan ('41). The diets used and the results are listed in tables 1 and 2.

### DISCUSSION

### A. Raw soybean protein

From the results of experiments with raw soybeans as given in table 1 the conclusion seems inescapable that lack of available methionine is the chief growth-limiting deficiency in unheated soybean protein.

The rates of gain observed in the case of raw soybean diets unsupplemented with methionine were from 2 to 3% per day.

TABLE 1

*Supplements to the raw soybean diet.[1] Relation of the supplements to the chick percentage gain.*

| DIET NO. | CHOLINE CHLORIDE | l-CYSTINE | dl-METHI-ONINE | GELATIN | GLYCINE | PERCENT-AGE GAIN PER DAY |
|---|---|---|---|---|---|---|
| 1 | 0.10 | ... | ... | 6 | ... | 2.8 |
| 2 | 0.40 | ... | ... | 6 | ... | 2.1 |
| 3 | 0.10 | 0.20 | ... | 6 | ... | 2.8 |
| 4 | 0.10 | ... | 0.25 | 6 | ... | 5.9 |
| 5 | 0.10 | ... | 0.50 | 6 | ... | 6.9 |
| 6 | ... | ... | ... | .. | 0.50 | 3.1 |
| 7 | 0.10 | ... | ... | .. | 0.50 | 3.2 |
| 8 | 0.10 | 0.16 | ... | .. | 0.50 | 3.4 |
| 9 | 0.10 | ... | 0.25 | 5 | ... | 6.6 |
| 10 | 0.10 | 0.20 | ... | 5 | ... | 2.0 |

[1] Expressed in per cent of diet.

The addition of choline (diets 6 and 7) or of l-cystine (diets 1 and 3, 7 and 8) produced little or no increase in this rate of gain. On the other hand, the addition of dl-methionine resulted in a striking increase in the rate of gain (diets 4, 5, and 9).

The rates of gain observed on the diets not supplemented with methionine, by comparison with similar rates obtained with purified diets containing arachin as the protein source (unpublished data), would indicate that methionine in the raw soybean protein is not entirely unavailable. The available quantity would seem roughly comparable to that in arachin; namely, approximately 0.6% of the protein.

The optimal level of supplementary dl-methionine for raw soybean protein appears to lie between 0.25 and 0.50% of the diet. This level permits a rate of gain which is comparable to that obtained on practical diets of the same total crude protein level; it is also equivalent or slightly superior to that observed in the case of heated soybean protein (table 2) without a methionine supplement.

## B. *Heated soybean protein*

Because of the more practical aspects of the heated soybean protein, more experimentation was devoted to a study of the protein value of soybean meal made by a controlled heating process. The results given in table 2 show that this type of soybean protein, when unsupplemented, was capable of supporting rates of gain not far below the standard 7% (diets 11 and 23). The addition of gelatin did not appreciably affect this rate of gain. The addition of l-cystine (diets 17 and 18; 24 and 29) caused little or no increase in rate of gain.

The addition of choline (compare diets 12 and 13; 23, 24 and 25; and 26 and 27) resulted in small but definite increases in rate of gain. Choline chloride at a level of 0.4% of the diet appeared slightly detrimental to growth in each of three comparisons with a 0.1% level (diets 1 and 2; 13 and 14; 15 and 16). Choline was employed at this high level in order

to insure the presence of a sufficient amount of choline to methylate any homocysteine that might be present. A comparatively high level of choline is required for this process in the chick (Klose and Almquist, '41). The results obviously do not suggest the presence of any appreciable amounts of available homocysteine in either raw or heated soybean protein.

TABLE 2

*Supplements to the heated soybean meal diet.[1] Relation of the supplements to the chick percentage gain.*

| DIET NO. | CHOLINE CHLORIDE | l-CYSTINE | dl-METHIONINE | l-LYSINE | l-TRYPTOPHANE | l-ARGININE | GLYCINE | GELATIN | PERCENTAGE GAIN PER DAY |
|---|---|---|---|---|---|---|---|---|---|
| 11 | ... | ... | ... | ... | ... | ... | ... | .. | 6.5 |
| 12 | ... | ... | ... | ... | ... | ... | ... | 6 | 6.6 |
| 13 | 0.10 | ... | ... | ... | ... | ... | ... | 6 | 7.2 |
| 14 | 0.40 | ... | ... | ... | ... | ... | ... | 6 | 6.7 |
| 15 | 0.10 | ... | 0.13 | ... | ... | ... | ... | 6 | 7.8 |
| 16 | 0.40 | ... | 0.13 | ... | ... | ... | ... | 6 | 7.4 |
| 17 | 0.10 | ... | 0.13 | ... | 0.10 | ... | ... | 5 | 7.7 |
| 18 | 0.10 | 0.28 | 0.13 | ... | 0.10 | ... | ... | 5 | 7.6 |
| 19 | 0.10 | 0.28 | ... | ... | 0.10 | ... | ... | 5 | 7.1 |
| 20 | 0.10 | 0.28 | 0.13 | ... | ... | ... | ... | 5 | 7.9 |
| 21 | 0.10 | 0.28 | 0.13 | ... | 0.10 | 0.40 | 1.00 | .. | 7.4 |
| 22 | 0.10 | 0.28 | 0.13 | 0.30 | 0.10 | 0.40 | 1.00 | .. | 8.1 |
| 23 | ... | ... | ... | ... | ... | ... | 0.50 | .. | 6.5 |
| 24 | 0.10 | ... | ... | ... | ... | ... | 0.50 | .. | 7.0 |
| 25 | 0.20 | ... | ... | ... | ... | ... | 0.50 | .. | 7.2 |
| 26 | ... | ... | 0.20 | ... | ... | ... | 0.50 | .. | 7.6 |
| 27 | 0.10 | ... | 0.20 | ... | ... | ... | 0.50 | .. | 7.9 |
| 28 | ... | 0.16 | ... | ... | ... | ... | 0.50 | .. | 7.3 |
| 29 | 0.10 | 0.16 | ... | ... | ... | ... | 0.50 | .. | 7.3 |

[1] Expressed in per cent of diet.

The small but distinct growth-increasing effect of choline is probably explainable on the basis of a methionine-sparing action. It seems unlikely that the basal diets could have been deficient in choline, since it has been shown that soybean meal at levels appreciably lower than those employed in the present work is a fully effective source of choline for the chick (Jukes, '41).

That heated soybean protein is slightly sub-optimal in methionine content is shown by the fact (diets 13 and 15; 19 and 20; 24 and 27) that a distinct increase in rate of gain took place in every case when dl-methionine was added. If it is assumed that all of the methionine (2.1%) in the heated soybean protein is available and that the added dl-methionine is fully effective, the optimal dietary methionine level, in the presence of added choline and cystine, would be approximately 0.55%.

The results with diets 17, 18, 19 and 20 indicate that there is no appreciable deficiency of tryptophane in heated soybean protein as far as the requirements of the chick are concerned. Diet 21 as compared to diet 22 furnishes an indication of a slight lysine deficiency for the highest rate of gain; this indication, however, is not supported by the results with diets 26 and 27 which yielded very satisfactory rates of gain in the absence of a lysine supplement.

It is evident that all other amino acids required in the diet of the chick are well provided by heated soybean protein at a level of 20%. It is further evident that the non-protein portion of the meal must be well utilized by the chick, since the diets used in this work contained as much as 30% of the fat, carbohydrate, and other non-protein components of the soybean.

The high rates of gain observed in the case of many of the diets suggest that 20% of heated soybean protein plus a small supplement of methionine constitutes at least as good an amino acid source for the chick as is found in either a typical practical diet of the same total crude protein level, or in several combinations of other proteins in a similar purified diet (Almquist and Mecchi, '42).

The rates of gain obtained with the raw soybean protein supplemented with gelatin, choline and methionine fell slightly but distinctly short of the values obtained with correspondingly supplemented heated soybean protein. This would indicate an improved general plane of nutrition with the latter product, as suggested by Hayward and Hafner ('41).

While our results seem in some ways to differ from those of Hayward and Hafner, it should be recalled that their diets for chicks contained approximately 9% of soybean protein, either raw or heated, out of a total protein level of 20% or more. It is entirely possible that the non-soybean proteins in the raw soybean diet may have so affected the composition of the total dietary protein as to present the condition of a positive but sub-optimal level of methionine in the diet. Our results with raw soybean protein alone in the diet indicate that an acute methionine deficiency exists, while there is little or no evidence for an uncomplicated cystine deficiency.

## SUMMARY

1. Raw and heated soybean protein were studied as the sole source of protein in a chick diet.

2. The principal growth-limiting deficiency in raw soybean protein is that of methionine.

3. Heated soybean protein is slightly deficient in methionine for the chick at the 20% protein level, but is complete in respect to all other amino acids required by the chick.

## LITERATURE CITED

ALMQUIST, H. J., AND E. MECCHI 1942 Glycine requirement of the chick. Proc. Soc. Exp. Biol. Med., vol. 49, p. 541.

HAYWARD, J. W., AND F. H. HAFNER 1941 The supplementary effect of cystine and methionine upon the protein of raw and cooked soybeans as determined with chicks and rats. Poultry Sci., vol. 20, p. 139.

JUKES, T. H. 1941 The effect of certain organic compounds and other dietary supplements on perosis. J. Nutrition, vol. 22, p. 315.

Klose, A. A., and H. J. Almquist 1941 Methionine in the diet of the chick. J. Biol. Chem., vol. 138, p. 467.

McCarthy, T. E., and M. X. Sullivan 1941 A new and highly specific colorimetric test for methionine. J. Biol. Chem., vol. 141, p. 871.

# BODY FATS IN RAT ACRODYNIA [1,2]

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An accompanying report of this series (Quackenbush et al., '42) dealt with the multiple nature of the deficiency which causes rat acrodynia. The acute symptoms which developed in animals fed a diet free from unsaturated fat were cured either by linoleic acid or by the combined effects of pyridoxine, pantothenic acid and an unidentified water-soluble factor present in a rice bran concentrate.[3] While linoleic acid was unique in curing the acrodynia when fed alone, the water-soluble factors as a group cured the acrodynia and restored growth. However, for the complete cure of the chronic dermatitis, manifested only by a scaliness of the feet and tail, both the water-soluble factors and linoleic acid were required.

In view of these results the question arose whether the water-soluble factors cured acrodynia by altering some phase of fat metabolism; for example, by (a) promoting more efficient utilization of the unsaturated acids; (b) producing desaturation; or (c) effecting the synthesis of small amounts of linoleic acid or its physiological equivalent from carbohydrate or protein (Sinclair, '40). It seemed possible that changes in the course of fat metabolism would be revealed by a study of the amount and character of the fat present in the

[3] Vitab.

body. The view that water-soluble vitamins play a role in fat metabolism has been expressed by several workers. Thiamine and pyridoxine have been reported to affect the deposition of fat in the liver (McHenry, '37; Halliday, '38; McHenry and Gavin, '41). Pyridoxine has been suspected of functioning in the utilization of essential fatty acids (Birch, '38).

## EXPERIMENTAL

### Procedure

Animals were analyzed at different ages, viz., at weaning, at the time of development of severe acrodynia, and after the supplements had been fed for 3 weeks. The first two groups were normal weanlings produced on our stock diet, Diet S (Steenbock, '23), and a potato meal diet, Diet P (Quacken-

TABLE 1

*Status of rats when taken for analysis.*

| GROUP | BASAL DIET | SUPPLEMENT (DAILY FOR 3 WEEK PERIOD) | NO. OF RATS | AGE (WEEKS) | MEAN BODY WEIGHT | MEAN DERMAL INDEX |
|-------|------------|--------------------------------------|-------------|-------------|------------------|-------------------|
| 1 | S |  | 8 | 3 | 44 | 0 |
| 2 | P |  | 6 | 4 | 40 | 0 |
| 3 | 5 | None | 23 | 9–10 | 58 | 5 |
| 4 | 5 | Pantothenic acid 50 µg. | 6 | 12 | 50 | 13 |
| 5 | 5 | Pyridoxine        20 µg. | 10 | 12 | 59 | 6 |
| 6 | 5 | Pantothenic acid 50 µg. Pyridoxine        20 µg. | 5 | 12 | 82 | 3 |
| 7 | 5 | Rice Bran Concentrate [1]        200 mg. | 17 | 13 | 101 | 2 |
| 8 | 5 | Ethyl linolate   10 mg. | 4 | 13 | 56 | 1 |

[1] Vitab.

bush et al., '39), respectively. The third group consisted of acrodynic animals of uniform weight with a dermal index of 5 to 6 which had received the acrodynia-producing diet [4], Diet 5 (Quackenbush et al., '39), after having been raised to weaning age on diet P. Groups 4 to 8, inclusive, had the same history as the third group except that they had received test supplements as described in table 1 for 3 weeks.

[4] Labor for the purification of the casein used in diet 5 was furnished by the Works Progress Administration.

The analyses consisted of determinations of amount and iodine values of body fat and liver fat. They were made by methods similar to those used by other workers. The rats were killed with ether, and the tissues then cut into small pieces and digested on a steam bath with 100 cc. of 30% alkali per 100 gm. of tissue. After 3 hours, one-fifth volume of alcohol was added and the digestion continued for 1 hour. The hydrolysate, freed from bones, was poured into a separatory funnel, cooled under the tap and acidified with dilute hydrochloric acid (1:1). The fatty acids were extracted with 100 cc. of freshly distilled petroleum ether per 200 gm. of tissue. The solution was washed with water, centrifuged, and the solvent removed under reduced pressure. Control tests with added oleic acid gave quantitative recoveries. Iodine values were determined by the method of Yasuda ('31).

## Results of analyses

The data show that animals from diet S contained more than twice as much fat as those from diet P and, furthermore, that this fat was more highly unsaturated. These differences are of considerable significance in view of our earlier observations (Quackenbush et al., '39), that weanling rats when taken from diet P developed acrodynia in 4 to 5 weeks, while rats of approximately the same weight from diet S developed acrodynia in 8 to 10 weeks. In the former case the symptoms were acute; in the latter, the symptoms tended to assume the chronic form.

With the development of acute acrodynia the animals were found to have lost a large part of their body fat; however, the residual fat was more unsaturated than the original stores. This situation was not changed in 3 weeks by the feeding of either pantothenic acid[5] or pyridoxine.[5] However, when the two supplements were fed together, marked increases in weight and partial alleviation of the dermal symptoms were accompanied by a threefold increase in body fat, and a slight

[5] The pyridoxine, pantothenic acid and riboflavin were generously furnished by Merck and Company.

lowering of its iodine value. With a supplement of rice bran concentrate, which produced a greater growth response and cured the dermal lesions completely, the body fat was increased fivefold and the iodine value diminished to that of weanling rats on the potato diet. On the other hand, linoleic acid which produced no increase in body weight cured the acrodynia, increased the body fat and, curiously enough, decreased the iodine value slightly.

TABLE 2

*Changes in tissue fats of rats with acrodynia.*

| GROUP | DESCRIPTION OF GROUP | CARCASS FAT ACIDS | | | LIVER FAT ACIDS | | | TOTAL UNSAT'D ACIDS (CALC'D AS OLEIC GM.) | UNSAT'D ACIDS % OF TOTAL |
|---|---|---|---|---|---|---|---|---|---|
| | | Wt. | % | I.V.[1] | Wt. | % | I.V. | | |
| 1 | Weanlings S | 4.8 | 11.1 | 72 | .13 | 5.2 | 134 | 4.03 | 82 |
| 2 | Weanlings P | 2.1 | 5.6 | 66 | .16 | 6.6 | 94 | 1.65 | 72 |
| 3 | Acute acrodynic | 1.2 | 2.2 | 78 | .08 | 2.3 | 98 | 1.14 | 86 |
| 4 | Pantothenic acid [2] | 1.2 | 2.3 | 76 | .07 | 2.6 | 118 | 1.12 | 87 |
| 5 | Pyridoxine | 1.5 | 2.6 | 77 | .07 | 2.0 | 107 | 1.34 | 85 |
| 6 | Pantothenic acid and pyridoxine | 3.9 | 5.0 | 74 | .16 | 3.9 | 120 | 3.42 | 85 |
| 7 | Rice bran concentrate [3] | 5.9 | 6.2 | 67 | .14 | 2.8 | 99 | 4.52 | 75 |
| 8 | Ethyl linolate | 2.0 | 3.5 | 73 | .07 | 2.5 | 107 | 1.68 | 82 |

[1] Iodine value.

[2] Groups 4 to 8, inclusive, received the substance named in this column as a daily supplement for a 3-week period. See column three of table 1.

[3] Vitab.

In general, a high degree of unsaturation prevailed in the fats of acrodynic rats, and in all cases the lower values were those of normal groups, or groups which had been cured of acrodynia. This finding is open to a number of interpretations: (1) Since the acrodynic animal suffers from a deficiency of linoleic acid or the physiological equivalent commonly called "essential" fatty acid, it may tend to produce larger amounts of unsaturated fat in an attempt to supply its needs. (2) Pantothenic acid and pyridoxine may have effected a stimulation of the production of unsaturated acids; however, this would necessitate recognition of a counteracting effect of some other factor in rice bran concentrate. (3) As has been

shown by Sinclair ('35) certain tissues tend to hold tenaciously to the more highly unsaturated fatty acids during fasting or inanition. It is possible that the higher degree of unsaturation of the fat of pathological animals was a reflection of this tendency, and that the fat synthesis which followed the healing of the lesions and consequent improvement in general condition was of such a nature as to re-establish the normal degree of unsaturation of the body fat.

The trend of changes of the liver fats was similar to that of the body fats. Although some differences in percentages of fat were noted, none of the livers were fatty. Some increases in.iodine values were apparent in animals which had received pantothenic acid, pyridoxine and ethyl linolate, but not in those which had received rice bran concentrate.

### Non-curative effect of isolated fats on acrodynia

Since the available chemical methods seemed inadequate for the detection of small concentrations of essential fatty acids an attempt was made to determine the biological potency of the isolated fatty acids. Evans and Lepkovsky ('32) had shown that the methyl esters of fatty acids from normal rats were curative of fat-deficiency symptoms when fed in doses of 5 drops daily. Accordingly, the fatty acids from both the rice bran concentrate group (group 7) and from the acrodynic rats (group 3) were esterified. The esters from the former were fed to seven acrodynic rats, and those from the latter to three similar rats. No improvement resulted from the daily administration of 20 to 30 drops of either supplement; the severity of the symptoms increased and many of the animals died before the end of the 3-week assay period. It was apparent that little storage of essential acids had been effected by rats during the production of acrodynia, and that no marked synthesis had resulted from the feeding of rice bran concentrate.

### SUMMARY

During the development of acrodynia in rats on a low-fat diet, the crude fatty acids decreased in amount but increased in iodine number.

The non-curative single supplements, pyridoxine and pantothenic acid, produced no significant alterations in the quality or quantity of fat.

Supplements which cured or alleviated the dermal symptoms, viz., linoleic ester, rice bran concentrate, or pyridoxine plus pantothenic acid, produced increases in total fat and decreases in iodine number.

Fatty acids from acrodynic rats or from rats which had been cured with rice bran concentrate did not cure acrodynia.

The studies did not reveal the mechanism through which pyridoxine, pantothenic acid and linoleic acid protect against acrodynia.

### LITERATURE CITED

BIRCH, T. W. 1938 The relation between vitamin B₆ and the unsaturated fatty acid factor. J. Biol. Chem., vol. 124, p. 775.

EVANS, H. M., AND S. LEPKOVSKY 1932 Vital need of the body for certain unsaturated fatty acids. III. Inability of the rat organism to synthesize the essential unsaturated fatty acids. J. Biol. Chem., vol. 99, p. 231.

HALLIDAY, N. 1938 Fatty livers in vitamin B₆-deficient rats. J. Nutrition, vol. 16, p. 285.

McHENRY, E. W. 1937 Vitamin B₁ and fatty livers. J. Physiol., vol. 89, p. 287.

McHENRY, E. W., AND G. GAVIN 1941 The B vitamins and fat metabolism. IV. The synthesis of fat from protein. J. Biol. Chem., vol. 138, p. 471.

QUACKENBUSH, F. W., B. R. PLATZ AND H. STEENBOCK 1939 Rat acrodynia and the essential fatty acids. J. Nutrition, vol. 17, p. 115.

QUACKENBUSH, F. W., H. STEENBOCK, F. KUMMEROW AND B. R. PLATZ 1942 Linoleic acid, pyridoxine and pantothenic acid in rat dermatitis. J. Nutrition, vol. 24, p. 225.

SINCLAIR, R. G. 1935 The metabolism of the phospholipids. VII. Further evidence of the selection and retention of unsaturated fatty acids by phospholipids of animal tissues. J. Biol. Chem., vol. 111, p. 275.

———— 1940 Growth of rats on high-fat and low-fat diets, deficient in the essential unsaturated fatty acids. J. Nutrition, vol. 19, p. 131.

STEENBOCK, H. 1923 A satisfactory ration for stock rats. Science, vol. 58, p. 449.

YASUDA, M. 1931 The determination of the iodine number of lipids. J. Biol. Chem., vol. 94, p. 401.

# NUTRITIONAL STUDIES ON POWDERED CHICKEN FEATHERS

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Recent studies from this laboratory (Routh, '42) have indicated that, when properly supplemented, powdered wool can be utilized by the rat for growth. A similar study of the nutritive properties of powdered hen's feathers has since been completed. This keratin is essentially a waste product in the poultry industry and interesting commercial applications might therefore emerge from the demonstration of its utilization by the animal organism.

## EXPERIMENTAL

To insure uniformity, a large well-mixed batch of hen's feathers which had been carefully cleaned and defatted was used as the starting material. The feathers were reduced to a powder by the process previously described for powdered wool (Routh, '40). In vitro digestion with trypsin reached a maximum when the feathers had been ground by 400,000 revolutions of the ball mill. Several batches (300–400 gm. each) were therefore ground for this period and combined for use in the feeding tests. The powdered feathers thus prepared had an average percentage composition of nitrogen 15.52, sulfur 2.86, cystine 4.75 and ash 1.5.

The diets in which the feathers were incorporated had the following percentage composition: powdered feathers 15, starch 41.4, sucrose 15, agar 2, salt mixture (Hubbell, Mendel

and Wakeman, '37) 2.5, hydrogenated cottonseed oil [1] 19, cod liver oil 5, and choline hydrochloride 0.1. As a source of the vitamin B complex each animal received daily 40 mg. riboflavin, 40 mg. thiamine hydrochloride, 200 mg. nicotinic acid and 50 mg. of rice polish concentrate [2] incorporated into two pills fed approximately 12 hours apart.

Inasmuch as data in the literature seemed to indicate that the four amino acids found in suboptimal concentrations in wool were also low in feathers the need for these amino acids was tested by incorporating them in different combinations in the diets at the following levels: tryptophane 0.1%, methionine 0.6%, histidine (as the monohydrochloride) 0.4%, and lysine (as the dihydrochloride) 0.7%. These and other supplements replaced an equivalent amount of starch in the diet.

The powdered wool used for comparison was similar to that described earlier (Routh, '42) and the casein used as a supplement in some of the tests was a commercial product employed in other dietary studies in the laboratory.

The diets were fed ad libitum to young rats weighing between 44 and 71 gm. The rats were housed in individual false-bottomed cages. Food consumption and body weight were recorded every 4 days. Individual growth records are presented in figures 1–3.

### RESULTS

Moderate growth was produced when tryptophane, methionine, histidine and lysine (diet O, fig. 1) were added to the basal diet containing the powdered feathers (15F). When any one of these four amino acids was omitted (as indicated by its initial letter T, M, H, or L, in fig. 1) growth failed to occur. A definite decrease in weight was observed on diet H from which the histidine was omitted. The lysine-deficient diet (L) failed to support growth in contrast to previous observations of fair growth on a similar diet containing

[1] Crisco.

[2] Ryzamin B from Burroughs-Wellcome Co., Tuckahoe, New York; riboflavin and thiamine through the courtesy of Merck and Co.

powdered wool instead of the powdered feathers (Routh, '42). This suggests that hen's feathers probably contain less lysine. The average gain on diet O was 1.4 and 1.5 gm. per day in the two series of animals.

Since casein is cheaper and is relatively rich in the four amino acids used as supplements, its supplementing capacity was tested by adding it to the diet containing powdered feathers at levels of 3, 5, and 8%. Its supplementing ability when fed at an 8% level (FC8, fig. 2) was little greater than
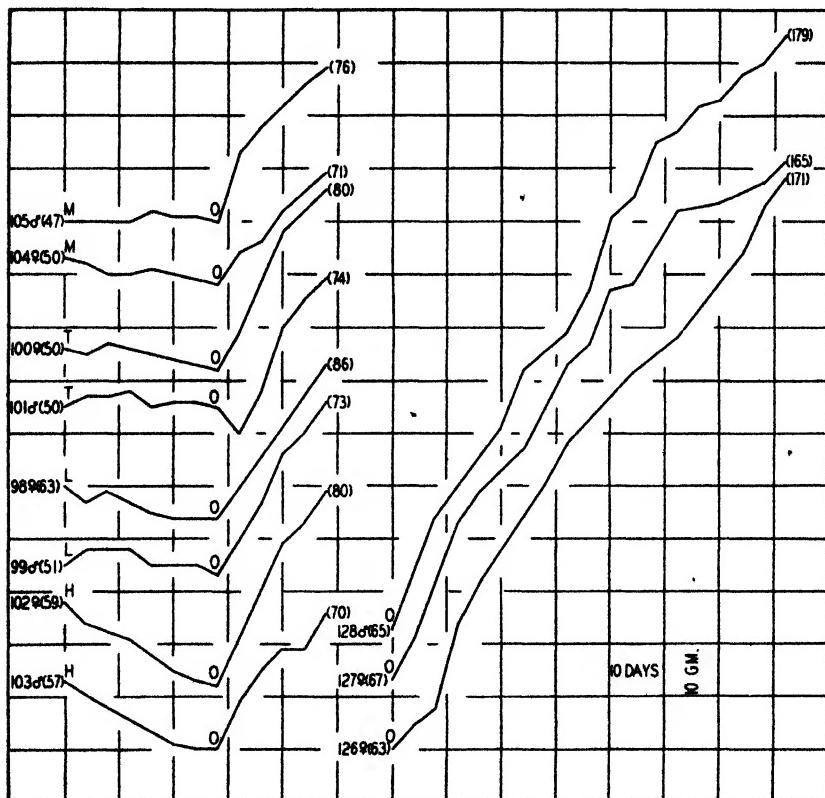


Fig. 1   Initial and final weights are given in parentheses. The various regimes and the food consumption per rat per day were as follows: O — diet 15F + 0.1% tryptophane, 0.6% methionine, 0.4% histidine, 0.7% lysine, 4.6 gm. (rats 98–105); 8.1 gm. (rats 126–128). M — diet O without the methionine, 3.0 gm. T — diet O without the tryptophane, 3.2 gm. L — diet O without the lysine, 4.1 gm. H — diet O without the histidine, 2.7 gm.

when fed at a 5% level (FC5, fig. 2). Average gains in weight
were 1.5 gm. and 1.3 gm. per day, respectively. When it was
fed at a 3% level (FC3, fig. 2) growth was decidedly inferior
(only 0.4 gm. per day).

From a careful estimate of the amounts of each of the
four amino acids present in the several diets it was concluded
that diets FC5 and FC3 might still be deficient in histidine
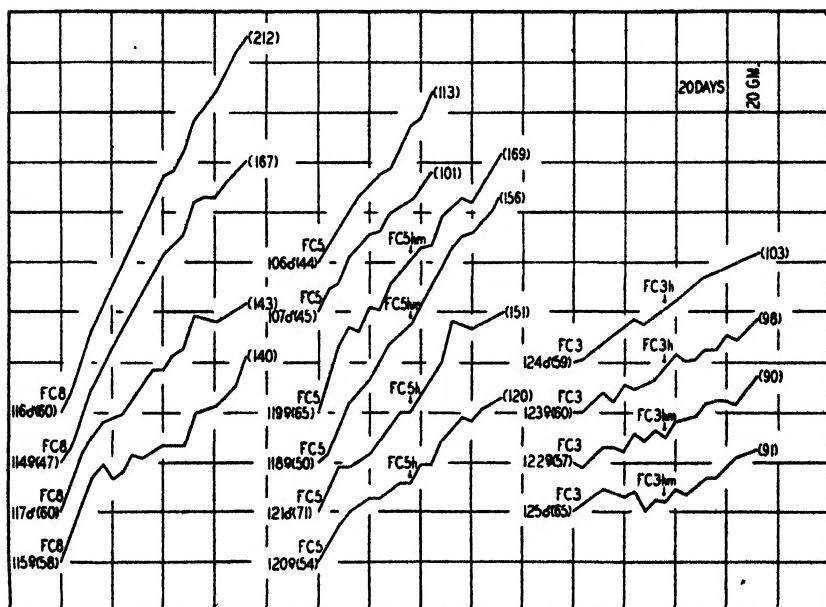


Fig. 2   Initial and final weights are given in parentheses. The various regimes
and the food consumption per rat per day are outlined below. F is the basal diet
containing 15% powdered feathers; C is casein and 3, 5 and 8 represent the per
cent of casein; h is 0.15% histidine and m is 0.15% methionine. FC8 — 6.9 gm.
(days 1–36), 7.8 gm. (days 37–72). FC5 — 6.3 gm. FC5hm — 8.0 gm. FC5h —
9.0 gm. FC3 — 4.8 gm. FC3h — 5.9 gm. FC3hm — 5.8 gm.

and possibly also in methionine. To determine the validity
of this conclusion these diets were supplemented at the end
of 36 days with either 0.15% histidine (FC 5h and FC3h) or
0.15% histidine plus 0.15% methionine (FC5hm and FC3hm)
(fig. 2). These additions made little difference in the growth of
rats on diet FC5 but increased appreciably the rate on diet

FC3. The average rate of growth on diet FC3 was 0.4 gm. per day and on FC3h and FC3hm 0.6 gm. per day. Since the growth rate did not approach that shown on the 5% level of casein it is obvious that there were also other limiting factors.
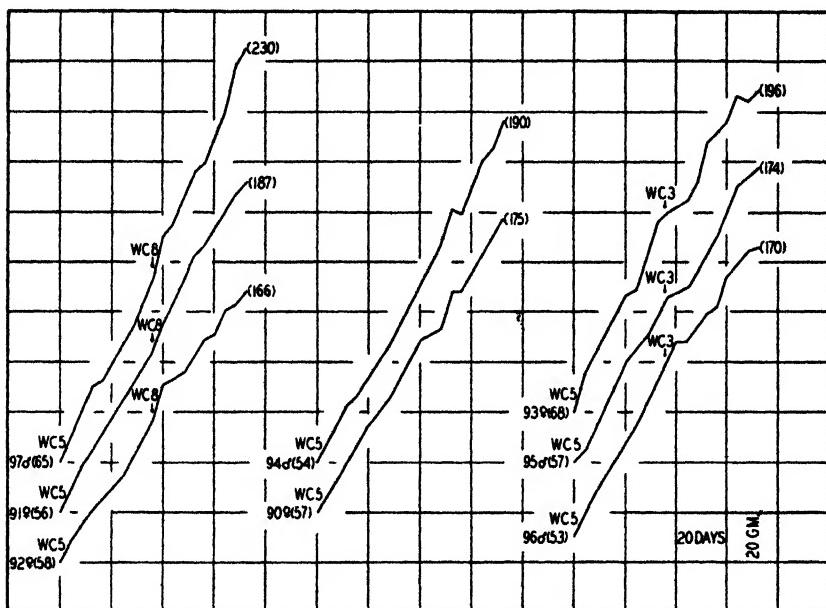


Fig. 3  Initial and final weights are given in parentheses. The various regimes and the food consumption per rat per day are outlined below. W is the basal diet containing 15% powdered wool; C is casein and 3, 5 and 8 represent the per cent of casein. WC5 — 7.1 gm. (days 1–36), 9.8 gm. (days 37–72). WC8 — 10.1 gm. WC3 — 9.0 gm.

The effect of supplementing diets containing powdered wool with casein is shown in figure 3. All of the animals in this group were placed on the basal diet containing 15% of powdered wool, modified by supplementation with 5% casein (diet WC5). After 36 days the supplement was changed to 3% casein or, in some instances, to 8%. The increase in weight of rats on these diets was more rapid than that observed in the rats on similar diets containing powdered feathers; the difference was especially striking on the diets containing 3%

of casein. The fact that wool is richer than feathers in histidine and lysine is probably responsible. Estimation of the amounts of these amino acids supplied by the diets suggests that histidine was the limiting factor. To judge from the similarity of growth rates, the utilization of the two powdered keratins was approximately the same in the diets containing 5 and 8% of casein.

The average food consumption on each diet is indicated in the explanation of the figures. In general it was roughly parallel to the growth rate.

Preliminary experiments with the hamster have given results very similar to those just described for the rat.

It appears that the protein requirement of domestic animals might be successfully met by feeding diets containing 15% of powdered feathers and 5% of casein; the commercial utilization of hen's feathers may thus be feasible.

### CONCLUSIONS

Powdered hen's feathers are capable of supporting moderate growth in the young rat when they are supplemented with tryptophane, methionine, histidine and lysine and fed in a diet otherwise adequate.

When 5 or 8% of casein was used as a supplement instead of these amino acids the growth rate was essentially the same. When 3% of casein was used the rats grew only about one-fourth as rapidly.

Powdered wool supplemented with 5 or 8% of casein gave practically identical results; with 3% of casein the powdered wool diet produced a definitely higher growth rate than the diet containing the powdered feathers.

### LITERATURE CITED

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, p. 273.

ROUTH, J. I. 1940 Chemical studies on powdered wool. J. Biol. Chem., vol. 135, p. 175.

———— 1942 Nutritional studies on powdered wool. J. Nutrition, vol. 23, p. 125.

# THE INFLUENCE OF THIAMINE, RIBOFLAVIN, PYRIDOXINE, AND PANTOTHENIC ACID DEFICIENCIES ON NITROGEN METABOLISM [1]

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While there is appreciable information concerning the influence of avitaminoses on fat and carbohydrate metabolism, the literature offers as yet relatively very little knowledge concerning nitrogen metabolism in vitamin deficiency diseases. This is particularly true with respect to the experimental rat.

In 1925 Morgan and Osburn reported on the effect of vitamin A deficiency on the nitrogen partition of the urine of the rat. In 1932 Sure and Kik found only a slight rise in non-protein nitrogen of the blood of the albino rat in a deficiency of the vitamin B complex. Recently Griffith and Mulford ('41) found that a deficiency of dietary choline and of the labile methyl supply in young rats produces a marked elevation of the non-protein nitrogen of the blood, which was coincident with renal hemorrhagic degeneration. The report of Lewinson ('38) on changes in non-protein nitrogen of the blood of dogs in B-avitaminosis cannot be critically reviewed, since his diets consisted of natural foods and were deficient in several dietary factors. Recently Schaefer, McKibbin and Elvehjem ('42) reported that in severe states of pantothenic acid deficiency in the dog there is a rise in the non-protein nitrogen of the blood.

The inadequate knowledge concerning the nitrogen metabolism in deficiencies of various components of the vitamin B complex and the recent development in this laboratory of procedures for blood (Sure and Wilder, '41) and urine analysis (Sure, Ford, Theis and Goldfischer, '41) stimulated this investigation, the results of which are presented in tables 1 to 5, inclusive. The partition of the non-protein nitrogen of the blood and urine was studied in thiamine, riboflavin, pyridoxine, and pantothenic acid deficiencies in the albino rat.

All of these studies were carried out with the paired feeding method of experimentation, according to the technique previously described (Sure and Dichek, '41). Since the litter mate control animals were restricted to the same amount of food consumed by the avitaminotic animals, the plane of nutrition was eliminated as a factor that possibly could have complicated the exogenous metabolism.

### DIETARY TECHNIQUE USED FOR PRODUCING DEFICIENCIES OF VARIOUS COMPONENTS OF THE VITAMIN B COMPLEX

The composition of all rations is given in table 1.

#### *Thiamine deficient diets*

The thiamine investigation was conducted on two types of diets. Ration 9 contained autoclaved yeast and beef as sources of the various components of the vitamin B complex other than thiamine. The control animals received in addition 20 µg. thiamine daily. This ration, supplemented occasionally with 0.05 to 0.1 µg. thiamine daily per animal, allowed prolonged maintenance, thus making it possible to produce a chronic state of deficiency. For this reason, urinary nitrogen excretions as well as blood changes in non-protein nitrogen constituents were studied on this as well as on the more purified ration 10. Each animal received daily as a supplement to ration 10 the following vitamin doses: 20 µg. riboflavin, 20 µg. pyridoxine, 0.5 mg. nicotinic acid, 6 mg. choline chloride, 100 µg. calcium pantothenate, and 150 mg. of rice

polish factor II (Supplee, Bender and Kahlenberg, '40). The control animals also received 20 µg. thiamine daily. All animals receiving no cod liver oil in their rations were given 2 drops halibut liver oil weekly as a source of vitamins A and D. A sufficiency of vitamin E for growth was furnished by the butterfat or hydrogenated cottonseed oil in the rations.

TABLE 1

*Percentage composition of rations.*

| COMPONENT | RATION | | | | | | |
|---|---|---|---|---|---|---|---|
| | 9 | 10 | 13 | 14 | 15 | 16 | 17 |
| Casein [1] | 16 | | 18 | | | | |
| Casein [2] | | 18 | | 18 | 18 | 18 | 18 |
| Agar-agar | | 2 | | 2 | | | |
| Agar-agar [3] | | | | | 2 | | |
| Nicotinic acid | | | | 0.05 | | | |
| Cod liver oil | | | 2 | | 2 | 2 | 2 |
| Butterfat | | 10 | | 10 | | | |
| Hydrogenated cottonseed oil [4] | 9 | | 8 | | 3 | 8 | 8 |
| Salts no. 351 [5] | 4 | | 4 | | | | |
| Salts no. 1 [6] | | 4 | | | | | |
| P. and H. salts [7] | | | | 4 | 4 | 4 | 4 |
| Autoclaved yeast [8] | 7 | | | | | | |
| Autoclaved beef [9] | 8 | | | | | | |
| Sucrose [3] | | | | | 71 | 68 | |
| Dextrin | 56 | 66 | | 65.95 | | | |
| Dextrin [10] | | | 68 | | | | |
| Cerelose | | | | | | | 68 |

[1] Supplied by Atterbury Bros., New York City, and purified by washing for 10 days with acidulated water and then by several washings with 25% ethanol at room temperature.

[2] Borden's purified casein, supplied under the trade name "Labco."

[3] Hot alcohol extracted.

[4] Crisco.

[5] Hubbell, Mendel and Wakeman ('37).

[6] Sure ('41).

[7] Phillips and Hart ('35).

[8] Fleischmann's, autoclaved for 5 hours at 15 pounds' pressure.

[9] Autoclaved for 6 hours at 20 pounds' pressure.

[10] Dextrin carried an 80% alcoholic extract of 25 gm. rice polishings in 100 gm. of ration.

## Riboflavin deficient diets

The riboflavin investigation was carried out on rations 13 and 14. The dextrin in ration 13, which is a modification of the diet of Day and Darby ('38), carried an 80% alcoholic extract of 25 gm. rice polishings per 100 gm. of ration as a source of the various components of the vitamin B complex with the exception of riboflavin. This diet was supplemented with 10 μg. thiamine and 10 μg. pyridoxine per animal per day and the control animals received in addition 20 μg. riboflavin daily. Ration 14 was supplemented with the same vitamin doses as ration 10 (previously outlined), the control animals being allowed 20 μg. riboflavin daily.

## Pyridoxine deficient diets

Pyridoxine deficiency was produced on ration 15, which is a modified diet of Antopol and Unna ('39). The daily vitamin supplements were the same as used by the latter investigators. Our experience with the production of pyridoxine deficiency in the rat corresponded with the recent observations of Lepkovsky and Krause ('42), that is, we encountered a mild avitaminosis, manifested by slow growth without the accompanying dermatitis. Substitution of the purified sucrose by commercial dextrose [2] did not change the character of the avitaminosis. Lepkovsky and Krause ('42) now find that, if weaned rats are first depleted for 10 to 12 days on a diet deficient in the vitamin B complex, a greater incidence of dermatitis can be produced later by pyridoxine deficiency. Since dermatitis is also produced by pantothenic acid deficiency (Richardson, Hogan and Itschner, '41) and riboflavin deficiency (Sure and Dichek, '41), it is questionable, as pointed out by Lepkovsky and Krause, if dermatitis or acrodynia is a reliable expression of uncomplicated pyridoxine deficiency. That we were actually dealing with an avitaminotic state was evident from the accelerated growth the control animals made when allowed food ad libitum.

[2] Cerelose.

## Pantothenic acid deficient diets

Pantothenic acid deficiency was produced on rations 16 and 17. As a supplement to this ration, the following daily vitamin doses were administered: 40 µg. thiamine, 40 µg. riboflavin, 40 µg. pyridoxine, 6 mg. choline chloride and 0.5 mg. nicotinic acid. The control animals received in addition 200 µg. calcium pantothenate daily. On ration 16, which is a modification of the diet by Unna ('40), only slow growth was the index of pantothenic acid deficiency, the characteristic symptoms described by Unna being absent. The change of ration 16 to ration 17, which consisted in the replacement of sucrose by dextrose,[3] together with different doses and kinds of vitamin supplements, resulted in the production of severe expressions of pantothenic acid deficiency. The daily vitamin supplements to ration 17 were as follows: 20 µg. thiamine, 20 µg. riboflavin, 20 µg. pyridoxine, 3 mg. p-aminobenzoic acid, 10 mg. inositol, 6 mg. choline chloride, and 0.5 mg. nicotinic acid. The control rats also received 100 µg. calcium pantothenate daily. Two to 3 weeks following the change from ration 16 to 17, the sores around the mouth, rusty spots on the fur and the ''blood-caked'' whiskers observed by Unna ('40) were regularly encountered. According to McElroy, Salomon, Figge and Cowgill ('41), the so-called bloody exudates of the nose which settle on the whiskers causing them to cake are composed not of blood but of coproporphyrin. After being on ration 17 for 6 to 7 weeks, animals collapsed suddenly when the plane of nutrition was quite adequate, an experience we have had recently with riboflavin deficiency (Sure and Dichek, '41). In this respect our experiences are in accord with those recently reported by Supplee, Bender and Kahlenberg ('42) on rats, and by Schaefer, McKibbin and Elvehjem ('42) on dogs. The sudden collapse may be due to adrenal hemorrhage and necrosis, and kidney and cardiac damage (Supplee and associates, '42; Mills et al., '40).

[3] See footnote 2, page 408.

With the aid of the Evelyn macro-micro photoelectric colorimeter (Evelyn, '37) a technique was developed for micro determinations of non-protein nitrogen, urea nitrogen, creatine and creatinine.  One milliliter of caudal blood was found sufficient for all the determinations of the non-protein constituents of the blood.  In order to study the effect of the severity of the vitamin deficiency state on the concentration of such blood constituents in the living animal, it was necessary to pool the blood from several pairs of animals subjected to the same treatment and showing similar symptomatology.  Enough blood could be secured by caudal bleeding from three animals weighing 60 to 70 gm.  Such a technique has made it possible for the first time to follow the nitrogen partition of the blood of the living small animal.  The details of such procedures are given elsewhere (Sure and Wilder, '41), but since the blood uric acid determination was not described, a brief outline is given for analysis of this constituent.[4]

This investigation involved a large number of blood and urine determinations.  Space, therefore, does not permit us to show detailed results.  All the blood data were grouped and averaged, and the urinary data were subjected to a statistical analysis according to Student's method, as outlined in the text by Paterson ('39).

NITROGEN METABOLISM IN THIAMINE DEFICIENCY

*Influence of thiamine deficiency on changes in non-protein constituents of the blood*

Blood samples were collected once weekly from six pairs of thiamine deficient animals and their controls on ration 9,

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[4] Blood uric acid determination.  To 1 ml. of the blood filtrate add 9 ml. of distilled water.  Add 5 ml. of 5% sodium cyanide.  Mix well and add 1 ml. of Benedict's uric acid reagent.  Mix and place centrifuge tubes in incubator at 40°C. for 45 minutes.  Place in ice bath for 15 minutes and then centrifuge for 5 minutes.  Decant and read in Evelyn macro photoelectric colorimeter, using a 660 filter.  From a previously calibrated curve obtained from various concentrations of pure uric acid crystals the values of blood uric acid are obtained.

from the sixth to the seventy-seventh day of vitamin depletion periods. No changes in the concentration of the non-protein constituents of the blood were apparent until the fortieth day. From then on there were 40 to 100% increases in the total N.P.N. and 30 to 50% increases in the urea nitrogen in the thiamine deficient animals. The changes in the uric acid were variable and in the creatine [5] and preformed creatinine [5] the differences were insignificant. In another group consisting of four pairs on ration 9 the blood changes were studied from the forty-second to the sixty-ninth day of vitamin depletion. From the forty-second day to the completion of the experiment there were 40 to 50% increases in the total N.P.N. and urea nitrogen, and about 24% increase in the preformed creatinine in the thiamine deficient rats. Similar observations were made on another group of four pairs of animals on ration 9 and on eleven pairs on ration 10. There were always marked increases in total N.P.N. and urea nitrogen and moderate increases in preformed creatinine in the avitaminotic rats, the earliest rise having been observed on the thirty-second day of vitamin depletion.

In order to obtain a more complete picture of the nitrogen metabolism, eleven pairs of animals were sacrificed in various states of thiamine deficiency, the summarized results of which are presented in table 2. It will be noted that even in the mild chronic state, and in the mild state of deficiency associated with small losses of body weight there are large increases in total N.P.N. and in urea nitrogen; also appreciable increases in preformed creatinine, the changes in uric acid and creatine being negligible. In the terminal states of avitaminosis, as indicated by polyneuritis and convulsions, only two pairs out of six showed increases in the total N.P.N. and urea nitrogen. The changes in the preformed creatinine were variable in this group. There were large increases in two avitaminotic animals, small increases in two other animals, and a decrease

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[5] The creatine is expressed as creatinine and is obtained by subtracting the creatinine values before hydrolysis from the total creatinine obtained after hydrolysis.

## TABLE 2

*Influence of various deficiences of the vitamin B complex on distribution of non-protein nitrogen of the blood of the albino rat.*

| AVITAMINOSIS | RATION | ANIMALS | NUMBER OF PAIRS | AVERAGE VITAMIN DEPLETION PERIOD | TOTAL NON PROTEIN NITROGEN | UREA | URIC ACID | PRE-FORMED CREATININE | CREATINE | AVITAMINOTIC STATE |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *mg /%* | *mg /%* | *mg /%* | *mg /%* | *mg /%* | |
| Thiamine | 9 | P[1] | 3 | 45 | 34.7 | 17.8 | 2.10 | 1.83 | 7.53 | Early vitamin depletion state |
| | | RC[2] | | | 32.0 | 9.0 | 2.72 | 1.80 | 6.75 | |
| Thiamine | 9 | P | 5 | 100 | 62.6 | 20.2 | 2.16 | 1.53 | 10.12 | Mild chronic state |
| | | RC | | | 48.2 | 16.8 | 2.11 | 0.98 | 9.82 | |
| Thiamine | 9 | P | 3 | 77 | 70.6 | 45.3 | 3.90 | 3.40 | 2.83 | Mild, slow loss of body weight |
| | | RC | | | 46.3 | 23.6 | 3.47 | 2.20 | 3.13 | |
| Thiamine | 9 and 10 | P | 6 | 98 | 69.3 | 30.0 | 2.54 | 2.28 | 6.11 | Polyneuritis and convulsions |
| | | RC | | | 63.8 | 28.8 | 2.89 | 1.97 | 6.81 | |
| Riboflavin | 13 and 14 | P | 16 | 113 | 57.7 | 19.3 | 1.72 | 1.38 | 5.19 | Advanced state of deficiency |
| | | RC | | | 48.4 | 15.3 | 1.93 | 1.62 | 5.00 | |
| Pyridoxine | 15 | P | 5 | 30th to 106th | 57.1 | 23.0 | 2.65 | 2.47 | 6.19 | Mild state of deficiency |
| | | RC | | | 51.2 | 18.8 | 1.93 | 2.58 | 3.92 | |
| Pyridoxine | 15 | P | 4 | 48th to 121st | 58.8 | 23.1 | 2.08 | 2.55 | 5.36 | Mild state of deficiency |
| | | RC | | | 48.1 | 20.2 | 1.69 | 2.43 | 3.94 | |
| Pantothenic acid | 16 and 17 | P | 6 | 61 | 43.0 | 20.0 | 2.35 | 2.80 | 3.25 | Advanced state of deficiency |
| | | RC | | | 47.5 | 21.5 | 1.90 | 2.65 | 3.95 | |
| Pantothenic acid | 16 and 17 | P | 4 | 68 | 49.0 | 19.5 | 2.10 | 2.50 | 3.25 | Terminal state of deficiency |
| | | RC | | | 38.0 | 14.5 | 2.75 | 2.15 | 4.00 | |

[1] P = pathological animal.
[2] BC = restricted control animal.

in one. During this period the plane of nutrition is reduced to almost complete starvation, which is also true for the control animals restricted to the same amount of food; therefore, it is possible that starvation may have complicated the blood picture.

*Influence of thiamine deficiency on partition of nitrogen of urine*

The partition of nitrogen in the urine was carried out on ten pairs of animals on diets 9 and 10 during metabolism periods ranging from 7 to 27 days, according to the technique recently described elsewhere (Sure, Ford, Theis and Goldfischer, '41). The urine was collected and analyzed daily, with the exception of Sundays. Monday's samples, therefore, represented 48 hours' excretion. Space does not permit reproduction of the numerous daily and bidaily records. Only average figures are consequently presented for all the animals covering the average vitamin depletion periods. The urinary data on riboflavin, pyridoxine, and pantothenic acid deficiencies were similarly treated. Since, with the exception of creatine, the advance of avitaminosis did not always show proportionate differences in the nitrogen constituents of urine, the average figures are representatives of marked changes, if any occurred regularly.

Because of differences in amounts of total nitrogen excreted, it was necessary, in order to make comparisons between thiamine deficient and control animals, to calculate the excretions of all nitrogenous constituents in the urine as percentage of total nitrogen. Therefore, the uric acid, preformed creatinine, and creatine figures were recalculated and expressed as uric acid nitrogen, preformed creatinine nitrogen, and creatine nitrogen.

It is evident from table 3 that thiamine deficiency is followed by large excretions of ammoniacal nitrogen and a marked creatinuria, as shown by both creatine and preformed creatinine elimination in urine. On diet 10 the creatine output

TABLE 8

*Influence of various deficiencies of the vitamin B complex on partition of nitrogen in the urine of the albino rat. Summarised average daily excretions per rat for all animals used in metabolism studies.*

(All the nitrogen constituents of the urine are expressed in milligrams.)

| CATEGORY OF INTEREST | | AVITAMINOSIS | | | |
|---|---|---|---|---|---|
| | | Thiamine | Riboflavin | Pyridoxine | Pantothenic acid |
| Ration | | 9 and 10 | 13 and 14 | 15 | 16 and 17 |
| Nos. of pairs | | 10 | 8 | 11 | 12 |
| Average metabolism period (days) | | 15 | 29 | 10 | 19 |
| Total nitrogen | P [1] | 114.9 | 89.6 | 131.8 | 103.9 |
| | RC [2] | 122.5 | 83.4 | 117.3 | 91.9 |
| Urea nitrogen | P | 84.6 | 65.0 | 107.5 | 76.3 |
| | RC | 95.2 | 61.9 | 92.4 | 66.4 |
| Per cent of T. N. [3] | P | 72.4 | 72.3 | 81.2, | 72.5 |
| | RC | 76.5 | 73.1 | 78.7 | 70.4 |
| Ammonia nitrogen | P | 6.9 | 5.8 | 4.5 | 8.7 |
| | RC | 3.2 | 3.7 | 4.7 | 8.1 |
| Per cent of T. N. | P | 6.6 | 6.7 | 3.5 | 8.4 |
| | RC | 3.1 | 4.6 | 4.1 | 9.4 |
| Allantoin nitrogen | P | 13.2 | 11.7 | 10.4 | 12.9 |
| | RC | 16.1 | 12.0 | 10.9 | 12.6 |
| Per cent of T. N. | P | 12.1 | 13.0 | 8.4 | 13.1 |
| | RC | 13.8 | 14.6 | 9.5 | 14.6 |
| Uric acid nitrogen | P | 0.63 | 0.59 | 0.51 | 0.84 |
| | RC | 0.63 | 0.58 | 0.56 | 0.79 |
| Per cent of T. N. | P | 0.55 | 0.67 | 0.39 | 0.84 |
| | RC | 0.61 | 0.69 | 0.48 | 0.94 |
| Preformed creatinine nitrogen | P | 2.44 | 0.54 | 0.97 | 0.83 |
| | RC | 1.54 | 0.69 | 0.96 | 0.82 |
| Per cent of T. N. | P | 2.11 | 0.60 | 0.77 | 0.83 |
| | RC | 1.23 | 0.82 | 0.81 | 0.95 |
| Creatine nitrogen | P | 1.30 | 0.56 | 0.91 | 0.65 |
| | RC | 1.04 | 0.76 | 0.72 | 0.63 |
| Per cent of T. N. | P | 1.16 | 0.63 | 0.69 | 0.62 |
| | RC | 0.81 | 0.91 | 0.61 | 0.69 |

[1] P = pathological or avitaminotic animal.

[2] RC = restricted control animal.

[3] T. N. = total nitrogen.

during the terminal stages of the thiamine deficiency was frequently found to be 100% greater than that in the litter mate control animals.

Since in the later stages of thiamine deficiency there is considerable catabolism of body tissue (Sure and Dichek, '41), during which periods there are considerable losses of creatine from muscle tissues, it was necessary to recalculate the excretions of creatine and preformed creatinine on the basis of

TABLE 4

*Summarized data on the influence of a deficiency of various components of the vitamin B complex on partition of nitrogen of the urine of the albino rat.*[1]

Nitrogen partition of urine expressed as per cent of total nitrogen.

| CATEGORY OF INTEREST | AVITAMINOSIS | | | |
|---|---|---|---|---|
| | Thiamine | Riboflavin | Pyridoxine | Pantothenic acid |
| Nos. of pairs | 10 | 8 | 11 | 12 |
| Urea nitrogen | | | | |
| Per cent decrease | 5.3 | 1.1 | | |
| Per cent increase | | | 3.2 | 3.0 |
| Ammonia nitrogen | | | | |
| Per cent decrease | | | 14.6 | 10.6 |
| Per cent increase | 113.2 | 45.7 | | |
| $\dfrac{D}{E_D}$[2] | 12.43 [3] | 3.80 [3] | 2.95 [4] | 1.17 |
| Allantoin nitrogen | | | | |
| Per cent decrease | 12.3 | 10.9 | 14.6 | 10.3 |
| $\dfrac{D}{E_D}$[2] | 0.54 | 3.80 [3] | 1.64 | 3.88 [3] |
| Uric acid nitrogen | | | | |
| Per cent decrease | 9.6 | 2.9 | 18.8 | 10.6 |
| $\dfrac{D}{E_D}$[2] | 0.37 | 0.68 | 4.09 [3] | 0.15 |

[1] Results are expressed as changes in the avitaminotic animals compared with litter mate controls.

[2] $\dfrac{D}{E_D}$ represents $t$ values which equal the mean difference divided by standard error of mean differences, according to Student's method of statistical analysis (Paterson, '39).

[3] Indicates that the experimental results are statistically very significant.

[4] Indicates that the experimental results are statistically significant.

100 gm. of body weight. Such figures eliminated the disturbing factor of changes in body weight influencing the creatine-creatinine metabolism. The average daily urinary excretions for the ten pairs of animals during the vitamin depletion periods were as follows: Preformed creatinine: avitaminotic, 2.42 mg.; control, 1.41 mg., or an increase for the thiamine deficient animals of 71.6%. Creatine: avitaminotic, 1.38 mg.; control, 1.03 mg., or an increase for the avitaminotic animals

TABLE 5

*Summarized data on the influence of a deficiency of various components of the vitamin B complex on creatine-creatinine excretion in urine, calculated on basis of body weights.*

Average daily excretions per 100 gm. body weight.

| AVITAMINOSIS | | NOS OF PAIRS | CREATINE NITROGEN | | | PREFORMED CREATININE NITROGEN | | |
|---|---|---|---|---|---|---|---|---|
| | | | Mg | Per cent increase | $\frac{D}{E_D}$ | Mg | Per cent increase | $\frac{D}{E_D}$ |
| Thiamine | P[1] | 10 | 1.38 | 34.9 | 1.47 | 2 42 | 71 7 | 8 60 [4] |
| | RC[2] | | 1.03 | | | 1.41 | | |
| Pyridoxine | P | 8 | 0.66 | 17.9 | 3.23 [3] | 0.75 | 25.0 | 2.46 [3] |
| | RC | | 0.56 | | | 0.60 | | |
| Riboflavin | P | 11 | 0.67 | 21.8 | 2.39 [3] | 0.81 | 5.2 | 1.09 |
| | RC | | 0.55 | | | 0.77 | | |
| Pantothenic acid | P | 12 | 0.55 | 25.0 | 3.51 [4] | 0.73 | 15.8 | 3.65 [3] |
| | RC | | 0.44 | | | 0.63 | | |

[1] Pathological animal.

[2] Restricted control animal.

[3] Indicates that the experimental results are statistically significant.

[4] Indicates that the experimental results are statistically very significant.

of 34.9%. When the results of creatine and creatinine excretions, as related to body weights, were subjected to Student's method of statistical analysis, only the large excretions of creatinine were found to be significant. The $\frac{D}{E_D}$ or $t$ value for one error in a thousand, when the number of variants is 10, is 3.169 (Paterson, '39). Actually, the $t$ value found was 8.601 (table 5), which is beyond any possibility of error.

The figure 1.465 for the creatine excretions indicates that, although the average figures show an increase of 34.9%, there were too many fluctuating results in obtaining such average, for the chances for error are one in about seven; hence, the results when correlated with fluctuations in body weight, are statistically insignificant. The $t$ value of 12.432 for 113.2% increase in excretion of ammonia (table 4) shows beyond any doubt that the results are free from any possibilities of error. On the other hand, the small decreases in allantoin and uric acid excretions have no statistical importance.

The marked excretions of preformed creatinine and retention of this constituent in the blood may be correlated with muscular atrophy during catabolism, as indicated by large losses of body weight in thiamine deficiency. The cause for the increases in both total N.P.N. and urea nitrogen is not as clear. The increases in blood total N.P.N. in choline deficiency (Griffith and Mulford, '41) and in pantothenic acid deficiency (Schaefer, McKibbin and Elvehjem, '42) are explained on the basis of kidney damage from hemorrhages. The question arises whether in thiamine deficiency there may be sufficient kidney damage to interfere with its normal excretory function. According to Hoobler ('28), one of the outstanding symptoms of infantile beriberi, as observed in the Philippines, is oliguria. There is a reduction in the amount of urine to the extent that the "diapers are wet once or twice a day and then with only scanty amounts of urine." Such symptomatology certainly should result in retention of non-protein constituents of the blood. In the rat, however, no noteworthy microscopic changes were found in the kidney in uncomplicated vitamin $B_1$ deficiency (Sure, Thatcher and Walker, '31). Since the diets used by the latter investigators carried autoclaved yeast which contains appreciable amounts of undestroyed thiamine, there may have been enough of this vitamin in the ration to prevent kidney damage. Further work on the influence of thiamine deficiency on the pathology of the kidney of the rat is, therefore, necessary to establish its relation to reten-

tion of total N.P.N. and urea nitrogen in the blood. On the other hand, the increase of these constituents of the blood in thiamine deficiency may very well be due to anhydremia produced in this avitaminosis (Sure and associates, '29 a, '29 b; Stucky and Rose, '29). It is possible that actually there may be a decrease in blood uric acid in thiamine deficiency, but being masked by the anhydremia, no noteworthy changes are apparent. Since in the majority of cases the increase in blood preformed creatinine was accompanied by a decrease in creatine, it is possible, as recently demonstrated by Bloch and Schoenheimer ('39) that the latter constituent was transformed into the former.

The marked increases in excretion of ammoniacal nitrogen may be explained by the attempt of the avitaminotic animals to neutralize acids developed in the acidosis associated with vitamin $B_1$ deficiency (Sure and Smith, '29).

### NITROGEN METABOLISM IN RIBOFLAVIN DEFICIENCY

*Influence of riboflavin deficiency on distribution of non-protein nitrogen constituents of the blood*

Eight pairs of rats fed ration 14 were sacrificed at various periods of riboflavin depletion ranging from 90 to 154 days, and five pairs on ration 13 were sacrificed after 74 to 107 days of vitamin depletion. The pathological changes observed were dermatitis, alopecia, keratitis, and premature senility. That these cases represented advanced stages of riboflavin deficiency is apparent from the observations that many control animals had gained two to three times as much body weight as the litter mate riboflavin deficient rats on the same amount of food intake. Yet, there were no noteworthy changes in the non-protein nitrogen constituents of the blood (table 2) when the results were subjected to a statistical analysis. The indications of slight rises in the total N.P.N. and in urea nitrogen have no significance, since the results for sixteen pairs of animals were inconsistent. An analysis of pooled blood from three pairs in terminal stages of riboflavin deficiency also showed no significant changes. One of the avitaminotic animals of this

group died the day following the termination of this experiment; another died 14 days following the end of this experiment. It may be concluded, therefore, that even in advanced and terminal stages of riboflavin deficiency there are no noteworthy changes in the various non-protein constituents of the blood.

### *Influence of riboflavin deficiency on partition of nitrogen of urine*

The partition of nitrogen in the urine was carried out on eight pairs of rats on ration 14, summarized average results of which are presented in table 3. The metabolism periods ranged between 8 and 72 days. In seven cases out of eight the period of study was not less than 16 days, and since the depletion periods were sufficiently advanced, the changes found are representative of what takes place in this avitaminosis. That there are large increases in excretion of ammonia is quite evident, and the results are statistically very significant, the possibility of error being less than one in a thousand. The figures on excretion of creatine and preformed creatinine are, however, misleading unless correlated with changes in body weight. For instance, Daniels, Hutton and Neil ('38), in the use of the creatinine-height coefficient as an index of nutrition of children, make use of the body weight. The creatinine coefficient in man is defined by Beard and Jacob ('39) ''as the amount in milligrams of creatinine or creatinine nitrogen excreted daily per kilogram of body weight.'' Since in the rat riboflavin plays a significant role in economy of food utilization (Sure and Dichek, '41), and frequently the control animals will weigh 100 to 150% more than the avitaminotic litter mates, lower excretion of both preformed creatinine and creatine may be anticipated in the riboflavin deficient rats. When calculated, however, on the basis of average daily excretions per 100 gm. body weight, the results were as follows: Preformed creatinine: avitaminotic, 0.66 mg.; control, 0.56 mg., or an increase of

17.9% for the riboflavin deficient animals. Creatine: avitaminotic, 0.75 mg.; controls, 0.60 mg., or an increase of 25% for the riboflavin deficient animals. Statistically, the results are significant for both the creatine and creatinine values. In the case of the former, the chances for error are about 5%. In the case of the latter, the chances for error are less than 1%. It may be concluded, therefore, that there is a moderate creatinuria in riboflavin deficiency in the albino rat. The large excretions in ammonia suggest an acidosis in this avitaminosis, as has been found in thiamine deficiency (Sure and Smith, '29). There is also a small but statistically significant reduction in allantoin excretion in this avitaminosis.

### NITROGEN METABOLISM IN PYRIDOXINE DEFICIENCY

*Influence of pyridoxine deficiency on distribution of non-protein nitrogen of the blood*

The blood changes in this avitaminosis were followed in five pairs of animals from the thirtieth to the one hundred and sixth day of vitamin depletion, according to the technique described earlier in the paper. As already stated, the only signs of pyridoxine deficiency observed were inadequate gains in body weight.

Although no regular changes were apparent either in the total non-protein nitrogen or urea nitrogen of the blood, the marked changes in both uric acid and creatine were consistent throughout the whole 76-day vitamin depletion period. This is also true of the next group, consisting of four pairs, the blood changes of which were followed from the forty-eighth to the one hundred and twenty-first day of pyridoxine deficiency. Averaging the results from these nine pairs of animals for a depletion period of 75 days, the avitaminotic rats showed an increase of 31.4% in blood uric acid and an increase of 47.0% in blood creatine. Whether retention of such blood constituents takes place earlier in pyridoxine deficiency we have not determined.

## *Influence of pyridoxine deficiency on partition of nitrogen in the urine*

The partition of nitrogen in the urine was carried out on two groups of animals. The first group consisted of five pairs, the urine of which was pooled. The second group consisted of six individual pairs. The results of these experiments are summarized in table 3. The pyridoxine deficient rats excreted daily an average of 0.91 mg. creatine while the control animals excreted 0.72 mg. or an increase of 26.4%. Since, however, the avitaminotic rats excreted daily more total nitrogen than the controls, calculated on the per cent of the total nitrogen, the increase of creatine excretion for the pyridoxine deficient animals is only 13.1%. When, however, the changes in body weight are taken into consideration and the average daily excretions are expressed on the basis of 100 gm. body weight, the results were as follows: Creatine: avitaminotic, 0.67 mg.; controls, 0.55 mg., or an increase for the pyridoxine-deficient animals of 21.8%. Preformed creatinine: avitaminotic, 0.81 mg.; controls, 0.77 mg., or an increase for the avitaminotic of 5.2%. Because of the retention of blood creatine, the origin of the urinary creatinine is most probably the blood creatine, the latter being derived from muscle metabolism. Such transformation is in line with the recent results of Bloch and Schoenheimer ('39). If pyridoxine is essential for normal muscle metabolism, it should prove of beneficial therapeutic use in certain types of myopathies. Two clinical reports show the therapeutic value of pyridoxine in such diseases (Antopol and Schotland, '40; Spies, Bean and Ashe, '39).

The reduction in allantoin excretion has no statistical value, since chances for error are about one in six.

The marked retention of blood creatine and uric acid and the reduction in urinary excretion of uric acid (table 4) suggest that there may be a disturbance in kidney function in pyridoxine deficiency.

NITROGEN METABOLISM IN PANTOTHENIC ACID DEFICIENCY

### Influence of pantothenic acid deficiency on distribution of non-protein nitrogen of the blood

The concentration of the non-protein constituents of the blood was followed in one group of three pairs of animals from the ninth to the forty-ninth day of vitamin depletion, and in another group of three pairs from the fourteenth to the forty-second day of vitamin depletion. The changes in these blood constituents were insignificant. In table 2 are submitted average results on animals changed from ration 16 to ration 17, which was followed by advanced states of deficiency, ending in the death' of several avitaminotic rats either the same day or several days after the last sampling of blood. One pair was sacrificed when the avitaminotic animal was in a dying condition. It will be noted that only in the terminal stages of pantothenic acid deficiency do we find a noteworthy rise in the total non-protein nitrogen and urea concentration of the blood. The changes in the rest of the blood constituents were negative. Schaefer, McKibbin and Elvehjem ('42) also found a marked rise in the total N.P.N. in severe states of pantothenic acid deficiency in the dog but none in mild states.

### Influence of pantothenic acid deficiency on partition of nitrogen of urine

Nitrogen partition of the urine was studied in twelve pairs of animals on rations 16 and 17. In a number of cases the changes in the urinary excretion of nitrogenous constituents were followed until the animals died. In this avitaminosis, as in the case of riboflavin deficiency, we have had complications of great differences in body weights between pathological and control animals. Calculated on the basis of 100 gm. body weight, the average daily excretions of creatine and preformed creatinine were as follows: Creatine: avitaminotic, 0.55 mg.; controls, 0.44 mg., or an increase for the pantothenic acid deficient rats of 25%. Preformed creatinine: avitaminotic, 0.73

mg.; controls, 0.63 mg., or an increase for the avitaminotic rats of 15.8%. The $t$ values indicate that the chances of error are less than one in one hundred for both the creatine and creatinine values. Hence, it may be concluded that in pantothenic acid deficiency there is a moderate creatinuria. There is also a small but statistically significant reduction in allantoin excretion in this avitaminosis.

The question arises whether specific gravity determinations of the blood would have been of assistance in the interpretation of the blood data. In the case of thiamine deficiency, this has already been done (Sure and associates, '28; Stucky and Rose, '29) and such correlations were made earlier in the paper. It is very doubtful, however, whether blood concentration or dilution complicated the picture of the non-protein constituents of the blood in riboflavin, pyridoxine, or pantothenic acid deficiencies for the following reasons: (a) In riboflavin deficiency the blood changes were negative, so information on concentration or dilution would have been of no importance; (b) in pyridoxine deficiency only two blood constituents increased, namely, uric acid and creatine, but not the main constituents, i.e., total N.P.N. and urea. If anhydremia were present, the latter constituents would have increased, and if edema were present, the same blood components would have decreased; (c) in pantothenic acid deficiency changes were found only in the terminal stages in two components. If either anhydremia or edema were present, the condition would have been evident much earlier during the progress of the avitaminosis.

### SUMMARY AND CONCLUSIONS

In thiamine deficiency there are large excretions of ammonia, creatine, and preformed creatinine in the urine. The creatinuria may be rated as marked. While there is a significant relationship between large excretions of preformed creatinine and body weight, there is no such definite relationship between creatine elimination and body weight in this avitaminosis when the data are examined statistically. There

is a great increase of total non-protein nitrogen and urea in the blood even in mild chronic states of thiamine deficiency.

In riboflavin deficiency there are large excretions of ammonia in the urine and a moderate creatinuria. The latter was manifested by excretions of both creatine and preformed creatinine when changes in body weight were taken into consideration during the vitamin depletion periods. There is also a small reduction in allantoin excretion in the urine. These changes were noted in advanced states of deficiency.

In pyridoxine there is a mild creatinuria, a reduction in urinary excretion of uric acid, and a marked retention of creatine and uric acid in the blood. These observations were made in mild states of this avitaminosis.

In pantothenic acid deficiency there is a moderate creatinuria, and a small reduction in allantoin excretion in the urine. The increases in total non-protein nitrogen and urea of the blood were noted only in the terminal stages of this avitaminosis.

### LITERATURE CITED

ANTOPOL, WM., AND C. E. SCHOTLAND 1940 The use of vitamin B₆ in pseudo-hypertrophic muscular dystrophy. J. Am. Med. Assn., vol. 114, pp. 1058–1059.

ANTOPOL, WM., AND K. UNNA 1939 Pathology of B₆ deficiency in the rat and response to treatment with 2-methyl-3-hydroxy 4,5-dihydroxymethyl-pyridine (vitamin B₆). Proc. Soc. Exp. Biol. and Med., vol. 42, pp. 126–127.

BEARD, H. H., AND E. J. JACOB 1939 Physiologic significance of the creatinine coefficient and the creatine tolerance test. A critical review. Arch. Neur. and Psychiatry, vol. 42, pp. 67–82.

BLOCH, K., AND R. SCHOENHEIMER 1939 Studies in protein metabolism. XI. The metabolic relation of creatine and creatinine studied with isotopic nitrogen. J. Biol. Chem., vol. 131, pp. 111–119.

DANIELS, A. L., M. K. HUTTON AND B. NEIL 1938 Relation of the creatinine-height coefficient to various indexes of nutrition. Am. J. Dis. Child., vol. 55, pp. 532–543.

DAY, P. L., AND WM. J. DARBY 1938 The influence of different casein preparations in riboflavin deficient diets upon the appearance of cataracts. Biochem. J., vol. 32, pp. 1171–1175.

EVELYN, K. A. 1937 Photoelectric microcolorimeter. J. Biol. Chem., vol. 115, pp. 63–76.

GRIFFITH, W. H., AND D. J. MULFORD 1941 Choline metabolism. VII. Some dietary factors affecting the incidence and severity of hemorrhagic degeneration in young rats. J. Nutrition, vol. 21, pp. 633–646.

HOOBLER, B. R. 1928 Symptomatology of vitamin B deficiency in infants. J. Am. Med. Assn., vol. 91, pp. 307–310.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, p. 273.

LEPKOVSKY, S., AND M. E. KRAUSE 1942 Dermatitis in pyridoxine-deficient rats. Proc. Soc. Exp. Biol. and Med., vol. 49, pp. 57–58.

LEWINSON, M. S. 1938 Stickstoffmetabolismus bei experimentell erzeugter B-Avitaminose. Ztschr. für Vitaminforsch., vol. 8, pp. 112–132.

McELROY, L. W., K. SALOMON, F. H. J. FIGGE AND G. R. COWGILL 1941 On the porphyrin nature of the fluorescent ''blood caked'' whiskers of pantothenic acid deficient rats. Science, vol. 94, p. 467.

MILLS, R. C., J. H. SHAW, C. A. ELVEHJEM AND P. H. PHILLIPS 1940 Curative effect of pantothenic acid on adrenal necrosis. Proc. Soc. Exp. Biol. and Med., vol. 45, pp. 482–484.

MORGAN, A. F., AND D. F. OSBURN 1925 The effect of vitamin A deficiency upon the character of nitrogen metabolism. J. Biol. Chem., vol. 66, pp. 573–593.

PATERSON, D. D. 1939 Statistical technique in agricultural research. McGraw-Hill Book Company, Inc.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., vol. 109, pp. 657–663.

RICHARDSON, L. R., A. G. HOGAN AND K. F. ITSCHNER 1941 Vitamin $B_6$, pantothenic acid and unsaturated fatty acids as they affect dermatitis in rats. Mo. Agr. Exp. Sta. Res. Bulletin 333, pp. 7–12.

SCHAEFER, A. E., J. M. McKIBBIN AND C. A. ELVEHJEM 1942 Pantothenic acid deficiency studies in dogs. J. Biol. Chem., vol. 143, pp. 321–330.

SPIES, T. D., WM. B. BEAN AND WM. F. ASHE 1939 A note on the use of vitamin $B_6$ in human nutrition. J. Am. Med. Assn., vol. 112, pp. 2414–2415.

STUCKY, C. J., AND WM. B. ROSE 1929 Studies in the physiology of vitamins. VII. Hemoglobin, solids, sugar and chloride changes in the blood of vitamin B deficient dogs. Am. J. Physiol., vol. 89, pp. 1–17.

SUPPLEE, G. C., R. C. BENDER AND O. J. KAHLENBERG 1940 The effect of complementing factors on the quantitative response and specificity of vitamin $B_6$. J. Nutrition, vol. 20, pp. 109–123.

———— 1942 Interrelated vitamin requirements: Kidney damage, adrenal hemorrhage and cardiac failure correlated with inadequacy of pantothenic acid. Endocrinology, vol. 30, pp. 355–364.

SURE, B. 1941 The existence of a new dietary factor essential for lactation. J. Nutrition, vol. 22, pp. 499–514.

SURE, B., AND M. DICHEK 1941 The sparing action of thiamin on body tissue catabolism. J. Nutrition, vol. 21, pp. 445–452.

SURE, B., Z. W. FORD, JR., R. M. THEIS AND M. GOLDFISCHER 1941 Nitrogen metabolism in hyperthyroidism. Endocrinology, vol. 28, pp. 806–815.

SURE, B., AND M. C. KIK 1932 Avitaminosis. VIII. Effect of vitamin B deficiency on concentration of non-protein nitrogen of blood. Proc. Soc. Exp. Biol. and Med., vol. 29, pp. 462–463.

SURE, B., M. C. KIK AND D. J. WALKER 1928 Vitamin requirements of nursing young. IV(b). Marked anhydremia associated with marked disturbance in hematopoietic function of nursing young suffering from vitamin B deficiency. Proc., Am. Soc. Biol. Chem., J. Biol. Chem., vol. 78, pp. xviii–xix.

————— 1929 a The effect of avitaminosis on hematopoietic function. II. Vitamin B deficiency. J. Biol. Chem., vol. 83, pp. 387–400.

SURE, B., AND M. E. SMITH 1929 b The effect of vitamin deficiencies on carbohydrate metabolism. II. The influence of uncomplicated vitamin B deficiency on concentration of the sugar, reducing non-sugar and alkaline reserve in the blood of the albino rat. J. Biol. Chem., vol. 84, pp. 727–740.

SURE, B., H. S. THATCHER AND D. J. WALKER 1931 Avitaminosis. I. Pathologic changes in nursing and in weaned albino rats suffering from vitamin B deficiency. Arch. Path., vol. 11, pp. 413–424.

SURE, B., AND V. M. WILDER 1941 Simplified photoelectric procedures for micro determination of non-protein nitrogen, urea nitrogen, creatine and creatinine of blood. The J. of Lab. and Clin. Med., vol. 26, pp. 874–878.

UNNA, K. 1940 Pantothenic acid requirement of the rat. J. Nutrition, vol. 20, pp. 565–576.

# INHIBITION OF THE SYMBIOTIC SYNTHESIS OF B COMPLEX FACTORS BY SULFONAMIDES

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Black, McKibbin and Elvehjem ('41) have found that the addition of sulfaguanidine to a B complex-free basal diet supplemented with the available crystalline components of the B complex, results in a depressed rate of growth of young rats. This effect was promptly ameliorated by feeding a liver extract, and could be prevented by feeding para-aminobenzoic acid. They suggest that sulfaguanidine inhibits the growth of certain microorganisms which may normally synthesize essential unidentified factors of the B complex. These factors were presumably supplied by the liver extract. Dann ('41) has also demonstrated that the incorporation of sulfaguanidine in the diet adversely affects the growth of rats fed no source of the B complex other than the crystalline vitamins.

Daft, Ashburn, Spicer and Sebrell ('42) have reported that rats fed a B complex-free diet containing 1% sulfaguanidine plus the crystalline factors of the B complex, develop hyaline sclerosis and calcification of the arteries. Whether the condition is due to the toxicity of the drug or to the deficiency of the unidentified B complex factors is not yet established.

The present study was designed to indicate whether the effect of sulfaguanidine on the growth of rats is due to a direct action on the metabolism of the animal or to an indirect

action through preventing a symbiotic synthesis of unidentified factors by the intestinal flora.

It seems probable that animals fed the B complex-free diet supplemented with the crystalline factors of the B complex are supplied with essential unidentified components of the B complex through synthesis by their intestinal flora. If this assumption is true, then the feces of these animals should contain these factors. Furthermore, if the effect of sulfaguanidine in the diet is to inhibit the growth of the organisms responsible for the synthesis of these factors in the intestinal tract, then the feces of such animals would be a poor source of the factors. On this premise we set up the following experiment.

### EXPERIMENTAL

White rats of the Sprague Dawley strain, weighing from 60 to 75 gm., were divided into twelve groups. The first four groups were fed the B complex-free basal diet [1] either alone or modified by the incorporation of certain sulfonamide derivatives as indicated in the table. Each animal was individually fed a supplement of crystalline vitamin having the following composition: thiamine 20 µg., riboflavin 40 µg., pyridoxine 20 µg., calcium pantothenate 100 µg., nicotinic acid 0.5 mg., inositol 1 mg., choline 5 mg., and biotin [2] 0.2 µg. per day.

The remaining eight groups were placed on the vitamin B complex-free basal diet containing 0.5% sulfaguanidine. These groups were depleted of the B complex vitamins for 2 weeks. At the end of this period they were all given the same daily supplements of crystalline vitamins as groups I to IV, and further supplementary feedings of feces or natural B complex sources as indicated in the table.

The feces from nine animals in each of the groups I to IV were collected and the feces from each group for each week were combined, dried at low temperature, and ground. Each

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[1] B complex-free basal diet having the following percentage composition: sucrose, 71; casein, 20; salts, 4; agar, 3; A, D, E oil, 2 (contains 888 U.S.P. units vitamin A, 177 U.S.P. units vitamin D, and 11 mg. alpha tocopherol per gram of oil).

[2] S M A — 1000 × concentrate.

week's collection was then divided into five equal parts, and one part was fed, by admixing with the basal ration, to each animal of the appropriate groups as indicated in the table. By this schedule a week intervened between the final collection of feces for the week and feeding the sample, during which time the feces were prepared for incorporating in the diet.

## RESULTS

The following table presents both the details of the experimental plan and the growth response of the groups.

TABLE 1

*The effect of sulfonamides on the symbiotic synthesis of a B complex factor(s).*

| GROUP NO | BASAL DIET | SUPPLEMENTS | NO OF ANIMALS | AVERAGE GAIN IN | |
|---|---|---|---|---|---|
| | | | | 4 wks. | 7 wks |
| | | | | No depletion period | |
| | | | | gm. | gm. |
| I | B complex-free + 0.5% sulfaguanidine | Pure B vitamins | 9 | 63 | 81 |
| II | B complex-free + 0.5% sulfanilamide | Pure B vitamins | 9 | 58 | 88 |
| III | B complex-free + 0.5% sulfadiazine | Pure B vitamins | 9 | 62 | 75 |
| IV | B complex-free | Pure B vitamins | 9 | 83 | 129 |
| | | | | After 2 weeks depletion | |
| V | B complex-free + 0.5% sulfaguanidine | Pure B vitamins + feces of group I | 5 | 63 | 92 |
| VI | B complex-free + 0.5% sulfaguanidine | Pure B vitamins + feces of group II | 5 | 88 | 124 |
| VII | B complex-free + 0.5% sulfaguanidine | Pure B vitamins + feces of group III | 5 | 76 | 102 |
| VIII | B complex-free + 0.5% sulfaguanidine | Pure B vitamins + feces of group IV | 5 | 99 | 138 |
| IX | B complex-free + 0.5% sulfaguanidine | Pure B vitamins + 250 mg. yeast | 8 | 106 | 152 |
| X | B complex-free + 0.5% sulfaguanidine | Pure B vitamins + 50 mg. yeast | 8 | 88 | 123 |
| XI | B complex-free + 0.5% sulfaguanidine | Pure B vitamins + 50 mg. yeast extract | 8 | 102 | 143 |
| XII | B complex-free + 0.5% sulfaguanidine | Pure B vitamins | 9 | 58 | 83 |

The effect of incorporating 0.5% sulfaguanidine in the diet
of animals receiving only the available pure components of
the B complex is apparent from the growth curves for groups I
and IV in figure 1. Feeding adequate amounts of yeast or
yeast extract to animals receiving a basal diet with 0.5%
sulfaguanidine permits a marked improvement in the rate of
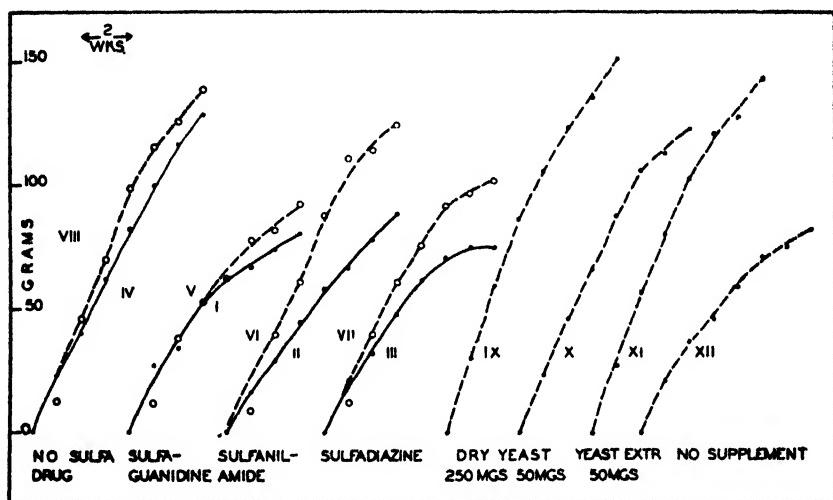


Fig. 1   The solid lines (.————.) indicate the growth of the undepleted groups
of animals fed all the pure vitamins except para-aminobenzoic acid and the B com-
plex-free diet containing the designated sulfonamides at a 0.5% level. Feces from
each group graphed on a solid line were fed, respectively, to each corresponding
group delineated on the adjoining broken line.

The broken lines (.------.) indicate the growth of the depleted groups receiving
the same pure vitamin supplements and the same basal diet containing 0.5%
sulfaguanidine. Further supplements of feces, as previously indicated, or of yeast
or yeast extract, were given in daily doses as indicated in the chart.

growth (cf. groups IX, X, XI, and XII). This result might
be attributed either to detoxication of the sulfaguanidine or
to supplementation with unidentified factors of the B complex.

A critical evaluation of the results presented in the first two
sets of curves (fig. 1) obtained with groups VIII, IV, V,
and I leads one to believe that the effect of sulfaguanidine is
to inhibit the growth of certain strains of the intestinal flora

which normally synthesize unidentified factors of the B complex.

Groups V and VIII received exactly the same diet (0.5% sulfaguanidine) and the same pure vitamin supplement as group XII, except that group V animals were fed in addition the feces from group I, and group VIII the feces from group IV. It is obvious from the growth curves that the feces from group IV animals contain factors which permit sulfaguanidine-fed animals to grow as well as those similarly fed but supplemented with yeast. On the other hand the feces from group I animals had little, if any, of these factors present.

The third and fourth pairs of curves in figure 1 present the results obtained by incorporating 0.5% sulfanilamide and sulfadiazine in the basal diets of groups II and III, respectively, and in feeding the feces of these two groups to the sulfaguanidine-fed animals in groups VI and VII, respectively. The responses indicate that both sulfanilamide and sulfadiazine have a toxic effect, but that neither is as effective as sulfaguanidine in inhibiting the growth of the particular organisms responsible for the synthesis of the unidentified B complex factors. The sulfanilamide apparently has less effect in inhibiting these particular organisms. Such a result might be anticipated from the data of Lawrence and Sprague ('41), whose study indicated that sulfanilamide was less effective in reducing fecal counts than was sulfaguanidine, sulfathiazole, or sulfapyridine.

The direct toxic effect of both drugs was further confirmed by our observations at autopsy. All of the animals fed 0.5% sulfadiazine or sulfanilamide exhibited marked hyperemia and enlargement of the thyroid which was more pronounced in the sulfadiazine groups, and the kidneys of a number of the animals revealed the yellow radiation on macroscopic examination. Histological examination revealed that these thyroids contained very little colloid material and the acini were hyperplastic. The thyroids of animals fed 0.5% sulfaguanidine were not enlarged and exhibited little evidence of hyperplasia.

Sections of the lung, heart, liver, kidney, stomach, pancreas, spleen, and cecum from several animals of each of groups I, IV, VIII, XI, and XII, as well as the sulfadiazine and sulfanilamide animals were also prepared and studied.

Examination of sections from animals fed sulfaguanidine did not reveal the hyaline sclerotic changes in the walls of the small arteries, which Daft et al. ('42) reported as occurring in young rats fed a B complex-free diet containing 1% sulfaguanidine and supplemented with pure vitamins for periods of 62 to 192 days. We did, however, observe small clear areas in the muscular coat of coronary arteries of most of the rats examined, and similar changes in the arteries of other organs. There was no evidence of inflammatory changes around the clear areas. These changes were observed in the tissues of the animals from group IV, which were not fed sulfaguanidine, as well as in the tissues of the animals receiving the diet with 0.5% sulfaguanidine. There was more amorphous basophilic material in the renal tubules of some of the animals receiving sulfanilamide and sulfaguanidine than in the controls. Findings on other tissues were essentially negative. All of the animals had received the experimental diet for 75 days at the time they were autopsied. Another lot of animals were sacrificed after 138 days on their respective diets and the histological picture remained the same.

### DISCUSSION

There is apparently a lack of correlation between the development of hyaline sclerosis, hyperplastic thyroids and the deficiency of the unidentified factors produced by feeding sulfonamides. Daft et al. ('42), feeding a diet containing 1% sulfaguanidine, produced a deficiency of the unidentified factor, and observed hyaline sclerosis and calcification of small arteries. According to the results of Mackenzie, Mackenzie and McCollum ('41), this diet should also have induced a hyperplasia of the thyroid, although there was no comment on either the presence or absence of these symptoms in the report by Daft et al. ('42).

In the present experiment animals fed diets with 0.5% sulfaguanidine with or without a deficiency of the unidentified factor did not develop either hyaline sclerosis of the arteries or hyperplasia of the thyroid. The animals fed 0.5% sulfanilamide or sulfadiazine did develop hyperplastic thyroids, but no hyaline changes in the arteries.

It is possible that the hyaline changes in the arteries are a specific effect of the 1% level of sulfaguanidine, or that some minor difference in the experimental procedure may account for the failure of our animals to develop hyaline sclerosis and calcification of the arteries.

The evidence seems clear that there are certain, as yet unidentified, factors present in yeast which are essential for the normal growth of rats, and normally synthesized in their intestines. The synthesis of these factors can usually be repressed by feeding the animals 0.5% sulfaguanidine in the diet. The sulfaguanidine apparently inhibits the growth of only certain types of organisms. Preliminary fecal counts, made after the animals had been fed a B complex-free diet containing 0.5% sulfaguanidine supplemented with the pure vitamins for 8 weeks, indicated that no notable decrease in the total count had been maintained, although there appeared to be a difference in the composition of the flora.

We have observed, in previous studies, that a certain percentage of individuals will grow well in spite of receiving a B complex-free diet containing 0.5% sulfaguanidine supplemented only with the crystalline components of the vitamin B complex. This observation suggests that certain species of organisms capable of synthesizing the unidentified factors, which are not commonly found in the rat intestinal flora, and which are not sensitive to sulfaguanidine, had become established in the intestines of these animals found refractory to the sulfaguanidine diet. Another possible explanation is that the critical strains, through conditioning, have become immune to the drug (Vivino and Spink, '42).

Woolley ('42) has demonstrated that the spontaneous cures of inositol deficiency symptoms in mice are due to the syn-

thesis of inositol by certain types of organisms in the intestinal flora. He found that the flora of mice which exhibit inositol deficiency symptoms does not synthesize appreciable amounts of this compound.

It has been shown by McElroy and Goss ('40) and others that the flora of ruminants synthesizes sufficient quantities of the B complex components to supply significant amounts of these factors to the host. There is evidence that the rat, equipped by nature with a large cecum, is supplied with certain factors of the B complex through intestinal synthesis, and that the mouse may be supplied with inositol in a similar manner. However, there are undoubtedly other species not so fortunately endowed by nature, which will not be able to rely on the intestinal flora to synthesize the essential factors and must obtain these factors in the diet. It is probable that dogs and man will be in the latter category, since neither has a large cecum or comparable "fermentation vat."

Further work on the types of organisms susceptible to sulfaguanidine therapy and a study of their ability to synthesize the unidentified factors will be helpful in elucidating this problem.

## SUMMARY

The inclusion of 0.5% sulfaguanidine in the diet of rats fed the available crystalline factors of the B complex results in a depressed growth. This effect can be counteracted by feeding such natural sources of the B complex as yeast and yeast extract. It is also prevented by feeding the feces of rats kept on the same diet without sulfaguanidine. These findings indicate that the sulfaguanidine inhibits the bacterial synthesis of essential factors which are present in the natural B complex.

## LITERATURE CITED

BLACK, S., J. M. McKIBBIN AND C. A. ELVEHJEM 1941 Use of sulfaguanidine in nutrition experiments. Proc. Soc. Exp. Biol. and Med., vol. 47, p. 308.

DAFT, F. S., L. L. ASHBURN, S. S. SPICER AND W. H. SEBRELL 1942 The occurrence of hyaline sclerosis and calcification of blood vessels in rats on sulfaguanidine. Pub. Health Reports, vol. 57, p. 217.

DANN, W. J.  1941  The synthesis of nicotinic acid by the rat.  J. Biol. Chem., vol. 141, p. 803.

LAWRENCE, C. A., AND K. D. SPRAGUE  1941  Effects of sulfanilamide, sulfathiazole and sulfanilylguanidine upon colon-typhoid-dysentery group. Proc. Soc. Exp. Biol. and Med., vol. 48, p. 696.

McELROY, L. W., AND H. GOSS  1940  A quantitative study of vitamins in the rumen contents of sheep and cows fed vitamin-low diets. I. Riboflavin and vitamin K. II. Vitamin $B_6$ (Pyridoxine). J. of Nutrition, vol. 20, pp. 527 and 541.

MACKENZIE, J. B., C. G. MACKENZIE AND E. V. McCOLLUM  1941  The effect of sulfanilylguanidine on the thyroid of the rat. Science, vol. 94, p. 518.

VIVINO, J. J., AND W. W. SPINK  1942  Sulfonamide-resistant strains of staphylococci: clinical significance. Proc. Soc. Exp. Biol. and Med., vol. 50, p. 336.

WOOLLEY, D. W.  1942  Synthesis of inositol in mice. J. Exp. Medicine, vol. 75, p. 277.

# POTASSIUM, SODIUM AND CHLORINE BALANCES OF PRE-SCHOOL CHILDREN RECEIVING MEDIUM AND HIGH PROTEIN DIETS [1]

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Sodium, potassium and chlorine are essential to the life of an organism. It is therefore surprising that there have been so few metabolism studies designed to determine the requirements of growing children for these elements. According to Stearns ('39) and also Shohl ('23, '39), sodium forms the largest portion of the total base of the body fluids and together with chlorine chiefly determines the osmotic equilibrium. Approximately 75% of the total body sodium functions in this manner and the remaining portion is found in a stable form in connection with bone and cartilage tissue. Chlorine occurs almost wholly in the extracellular fluids, the chief exception being the small amount contained in the red blood cells. Potassium functions mainly in relation to protein tissue, since approximately 72% of the total is contained in the muscle tissue. The remainder is found in other tissues, especially in the red blood cells and a small amount is present in the extracellular fluids. The assumption is generally made that all of the sodium and chlorine are contained in the extracellular and all of the potassium in the intracellular materials.

In previous publications, Hawks et al. ('37, '38, '40 and '42) reported studies showing the effect of increasing the protein

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content of the diet on the protein, caloric, calcium and phosphorus utilization of pre-school children. At the same time they also determined potassium, sodium and chlorine balances which will be discussed in this paper.

The details of the experimental procedure have been discussed fully in a previous publication (Hawks, Dye and Bray, '37), thus only brief details will be given here. Two groups of pre-school children received, first, a diet containing 3 and then one containing 4 gm. of protein per kilogram of body weight. In the first experiment, the 4-gm. protein diet contained additional milk, part of it skimmed to adjust the caloric value, meat and whole egg, while in the second experiment, egg white and gelatin were added and the amount of butter was reduced. All other foods comprising the two diets remained the same. In both experiments the conditions were very carefully controlled. The children consumed the food quantitatively and followed a definite routine each day. Experimental periods continued from 15 to 24 days. At the beginning of the experiment the three boys B, D, and V, were 55, 57 and 49 months old, and the two girls C and J were 37 and 38 months, respectively.

Sodium was determined by the Butler and Tuthill ('31) modification of the Barber and Kolthoff ('28, '29) uranyl zinc acetate method. Potassium was precipitated as sodium potassium sulfate according to the initial procedure of the Lindo-Gladding method (Peters and Van Slyke, '32). The potassium value was then determined by difference, using the sodium figures as previously determined. Chlorine was determined according to the Volhard-Harvey titration method (Peters and Van Slyke, '32).

Table 1 gives the balance data for all children on both experiments. The values are expressed as milligrams as well as milliequivalents per kilogram of body weight.

## TABLE 1

### Balance data expressed per kilogram of body weight per day

| EXPERIMENT CHILD | | PROTEIN IN DIET | POTASSIUM | | | | SODIUM | | | | CHLORINE | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Range | Mean | | Proportion of intake [1] | Range | Mean | | Proportion of intake [1] | Range | Mean | | Proportion of intake [1] |
| | | gm | mg | mg | meq | % | mg | mg | meq | % | mg | mg | meq | % |
| I B | Intake | 3 | 177–197 | 186 | 4.76 | — | 59.3–90.2 | 66.6 | 2.90 | — | 116–163 | 126 | 3.55 | — |
| | | 4 | 212–226 | 219 | 5.60 | — | 63.0–86.8 | 72.0 | 3.13 | — | 121–165 | 139 | 3.92 | — |
| | Feces | 3 | 26–43 | 32 | 0.82 | 17.3 | 0.4–1.9 | 1.1 | 0.05 | 1.6 | 2–3 | 3 | 0.08 | 2.2 |
| | | 4 | 26–42 | 36 | 0.92 | 16.2 | 0.2–0.9 | 0.6 | 0.03 | 0.8 | 3–4 | 3 | 0.08 | 2.4 |
| | Urine | 3 | 136–149 | 144 | 3.68 | 77.3 | 51.7–75.7 | 60.3 | 2.62 | 90.7 | 98–138 | 110 | 3.10 | 87.2 |
| | | 4 | 163–183 | 171 | 4.37 | 78.1 | 52.1–74.7 | 65.1 | 2.83 | 90.3 | 95–127 | 113 | 3.19 | 81.7 |
| | Apparent Retention | 3 | 2–20 | 10 | 0.26 | 5.4 | (−1.8)–12.9 | 5.2 | 0.23 | 7.7 | 3–23 | 13 | 0.37 | 10.6 |
| | | 4 | 2–20 | 12 | 0.31 | 5.7 | (−2.7)–16.3 | 6.3 | 0.27 | 8.9 | 13–34 | 23 | 0.65 | 15.9 |
| I D | Intake | 3 | 175–195 | 184 | 4.71 | — | 58.8–89.3 | 65.9 | 2.86 | — | 115–162 | 125 | 3.52 | — |
| | | 4 | 210–224 | 217 | 5.55 | — | 62.5–85.5 | 71.3 | 3.10 | — | 120–164 | 138 | 3.89 | — |
| | Feces | 3 | 23–30 | 26 | 0.67 | 14.3 | 0.1–1.8 | 0.7 | 0.03 | 1.0 | 2–4 | 3 | 0.08 | 2.2 |
| | | 4 | 21–29 | 25 | 0.64 | 11.5 | 0.0–0.7 | 0.2 | 0.01 | 0.3 | 2–3 | 3 | 0.08 | 1.9 |
| | Urine | 3 | 146–157 | 152 | 3.89 | 82.5 | 52.9–90.9 | 61.2 | 2.66 | 93.1 | 91–144 | 108 | 3.05 | 86.1 |
| | | 4 | 175–185 | 180 | 4.60 | 82.7 | 57.8–76.7 | 65.3 | 2.84 | 91.6 | 99–132 | 112 | 3.16 | 81.3 |
| | Apparent Retention | 3 | (−1)–11 | 6 | 0.15 | 3.2 | (−2.8)–7.6 | 4.0 | 0.17 | 5.9 | 8–21 | 14 | 0.39 | 11.7 |
| | | 4 | 5–26 | 12 | 0.31 | 5.8 | 1.0–10.0 | 5.8 | 0.25 | 8.1 | 16–29 | 23 | 0.65 | 16.8 |
| II V | Intake | 3 | 199–206 | 203 | 5.19 | — | 58.7–60.4 | 59.4 | 2.58 | — | 107–114 | 110 | 3.10 | — |
| | | 4 | 198–208 | 202 | 5.17 | — | 58.8–62.7 | 60.3 | 2.62 | — | 107–111 | 110 | 3.10 | — |
| | Feces | 3 | 27–34 | 31 | 0.79 | 15.2 | 0.2–00.4 | 0.3 | 0.01 | 0.5 | 1–3 | 2 | 0.05 | 1.8 |
| | | 4 | 25–34 | 29 | 0.74 | 14.5 | 0.2–00.5 | 0.3 | 0.01 | 0.6 | 2–3 | 2 | 0.05 | 1.9 |
| | Urine | 3 | 156–161 | 160 | 4.09 | 78.7 | 51.0–53.3 | 52.0 | 2.26 | 87.6 | 100–109 | 102 | 2.88 | 92.9 |
| | | 4 | 153–166 | 159 | 4.07 | 78.7 | 49.8–52.4 | 51.2 | 2.23 | 84.8 | 92–98 | 95 | 2.68 | 86.2 |
| | Apparent Retention | 3 | 10–17 | 12 | 0.31 | 6.1 | 5.4–8.9 | 7.1 | 0.31 | 11.9 | 2–11 | 6 | 0.17 | 5.3 |
| | | 4 | 9–21 | 14 | 0.36 | 6.8 | 6.8–11.4 | 8.8 | 0.38 | 14.6 | 9–16 | 13 | 0.37 | 11.9 |
| II C | Intake | 3 | 206–214 | 211 | 5.40 | — | 60.8–62.9 | 61.7 | 2.68 | — | 112–118 | 115 | 3.24 | — |
| | | 4 | 205–213 | 207 | 5.30 | — | 60.2–64.6 | 61.8 | 2.69 | — | 109–114 | 112 | 3.16 | — |
| | Feces | 3 | 24–42 | 32 | 0.82 | 15.1 | 0.2–0.5 | 0.3 | 0.01 | 0.6 | 1–2 | 2 | 0.05 | 1.5 |
| | | 4 | 26–30 | 28 | 0.72 | 13.2 | 0.4–0.5 | 0.4 | 0.02 | 0.8 | 2–3 | 2 | 0.05 | 2.1 |
| | Urine | 3 | 161–174 | 167 | 4.27 | 79.2 | 53.0–57.6 | 54.5 | 2.37 | 88.3 | 102–113 | 107 | 3.02 | 92.6 |
| | | 4 | 162–173 | 166 | 4.25 | 80.4 | 47.9–57.8 | 53.9 | 2.34 | 87.2 | 93–102 | 98 | 2.77 | 87.2 |
| | Apparent Retention | 3 | 4–22 | 12 | 0.31 | 5.7 | 5.1–8.8 | 6.9 | 0.30 | 11.1 | 3–13 | 6 | 0.17 | 5.9 |
| | | 4 | 9–19 | 13 | 0.33 | 6.4 | 5.0–12.4 | 7.5 | 0.33 | 12.0 | 7–18 | 12 | 0.34 | 10.7 |
| II J | Intake | 3 | 193–202 | 197 | 5.04 | — | 56.9–58.7 | 57.8 | 2.51 | — | 105–111 | 108 | 3.05 | — |
| | | 4 | 190–198 | 194 | 4.96 | — | 56.0–60.4 | 57.8 | 2.51 | — | 103–106 | 105 | 2.96 | — |
| | Feces | 3 | 32–38 | 35 | 0.90 | 17.6 | 0.7–2.1 | 1.6 | 0.07 | 2.6 | 3–4 | 3 | 0.08 | • 2.8 |
| | | 4 | 35–39 | 36 | 0.92 | 18.8 | 1.0–1.5 | 1.3 | 0.06 | 2.4 | 2–3 | 3 | 0.08 | 2.7 |
| | Urine | 3 | 147–154 | 151 | 3.86 | 76.6 | 46.4–52.6 | 50.9 | 2.21 | 88.0 | 90–104 | 99 | 2.80 | 91.6 |
| | | 4 | 145–153 | 148 | 3.78 | 76.2 | 48.2–51.5 | 49.9 | 2.17 | 86.4 | 89–93 | 90 | 2.54 | 86.2 |
| | Apparent Retention | 3 | 8–17 | 11 | 0.28 | 5.8 | 3.4–11.6 | 5.3 | 0.23 | 9.4 | 1–18 | 6 | 0.17 | 5.6 |
| | | 4 | 6–13 | 10 | 0.26 | 5.0 | 4.1–7.6 | 6.6 | 0.28 | 11.2 | 11–13 | 12 | 0.34 | 11.1 |

[1] Percentage calculations were based on total daily figures in order to eliminate errors caused by rounding out the kilogram figures.

## Potassium

*Experiment I.* Increasing the protein content of the diet from 3 to 4 gm. per kilogram had little, if any, effect on the apparent potassium retention for subject B, but caused an increase in the retention for child D. The slightly higher intake values on this diet may have been a factor in producing the increased retention. Fecal values were practically the same on the two diets, which made the amount of potassium absorbed higher in proportion to the intake on the 4-gm. protein diet. The increase in urinary potassium was also related to diet, for the percentage of the intake thus eliminated remained the same.

*Experiment II.* In this experiment the increase in the protein content of the diet apparently had no effect on potassium utilization. The mean intake values remained the same for individual children on the two diets and there was little variation in the figures from period to period. Fecal and urine values both remained practically the same for each child and there was no significant change in the apparent retention.

## Sodium

*Experiment I.* Although there was a slight increase in sodium intake on the 4-gm. protein diet, the higher sodium retentions were probably due to the increase in the protein content of the diet because both milligrams of sodium and percentages of the intake retained increased. The children absorbed approximately 99% of the intake on both diets, but a smaller proportion of the intake was eliminated in the urine on the 4- than on the 3-gm. protein diet.

*Experiment II.* In this experiment the higher protein diet also caused an increase in the milligrams of sodium and in the proportion of the intake retained. There was little range in intake values and the mean figures were almost identical for each child. Feces contained practically no sodium so the differences in utilization on the two diets occurred after the sodium entered the body and were manifest by a lower amount and proportion of sodium excreted in the urine on the 4-gm.

protein diet. The higher fecal sodium for subject J no doubt was due to the fact that she had rather loose stools during the entire time.

## Chlorine

*Experiment I.* The increase in the protein content of the diet caused a decided increase in chlorine retention in spite of the fact that the mean chlorine intake was slightly higher on the 4- than on the 3-gm. protein diet. Fecal outputs remained the same on the two diets, but the proportion of the intake eliminated in the urine diminished on the higher protein diet.

*Experiment II.* Chlorine intake was exceedingly constant on both diets, all figures ranging.between 102 and 118 mg. per kilogram. The change in the protein content of the diet caused no difference in fecal outputs, but urine values decreased for each child and represented a smaller proportion of the intake on the higher protein diet. Thus without a doubt the higher protein diet caused an increase in the amount of chlorine retained.

### DISCUSSION

Shohl ('39) states that in balance studies with sodium and chlorine it may take 2 weeks to establish equilibrium even on a constant diet and fluid intake. The data in the present experiment indicate that the children were in equilibrium. The fluid intakes and the diets, with the exception of the change in protein content, were constant throughout the entire experiment. The 10- or 12-day preliminary periods immediately preceding the medium protein diet were apparently adequate because there was little variation between the values during each 3-day period, and no more variation during the first periods than between those later in the experiment. In the second experiment, 9 days of the higher protein diet were considered as a preliminary period, but the average values for this period were almost identical with the average values during the last 15 days, and there was no more variation in

the results during the first 3-day period following the change
in the protein content than there was during the later periods.
It must be remembered, however, that there were practically
no changes in sodium and chlorine intakes on the higher pro-
tein diets, and thus the change probably did not disturb sodium
and chlorine equilibrium. Therefore it seems that any con-
clusion drawn from the results would be valid within the
limits of any balance study.

Losses through the skin, which were not determined, prob-
ably reduced the quantity of these three minerals actually
retained in the body. No figures are available for children
of pre-school age, but Swanson and Iob ('33) found that in-
fants from 3 to 6 months of age lost a considerable proportion
of the apparent retention in this manner. The babies lost from
20 to 38% of the potassium, from 9 to 14% of the sodium,
and from 13 to 18% of the chlorine. The children in the pres-
ent study probably had relatively constant losses from day to
day because the conditions of the experiment were so care-
fully controlled. They followed a regular routine for activity
and rest, and the amount of clothing was varied to prevent
excessive perspiration.

Since body tissue as a whole contains more potassium than
sodium, the difference increasing with age, it seems desirable
that the diet for growing children should contain a similar
proportion. The Na/K ratio in milk is 1/1.5 by equivalents
and should represent a desirable ratio for growth. In this
study all of the diets had Na/K ratios similar to or higher
than that for milk. In the first experiment, these ratios were
1/1.6 and 1/1.8 on the 3- and 4-gm. protein diets, respectively,
and in the second experiment, they were 1/2.0 on all diets.
Since the relationship was the same or nearly so, on the two
levels of protein intake, the proportion could not have influ-
enced the retention of these minerals.

In their studies on children, Macy and her associates
(Souders, Hunscher, Hummel and Macy, '39; Hummel,
Hunscher and Macy, '39; Hunscher, Hummel and Macy, '40)
reported balance data for potassium, sodium and chlorine.

Intakes, as compared to those in the present study, showed a greater range in values and were lower for potassium, higher for sodium and similar for chlorine. In spite of these differences in intake, the children in both studies eliminated similar percentages of the intake of these three minerals in the urine and feces, and the retention figures were all within a similar range.

The actual retentions for the children in the present study ranged slightly above and below the figures for the daily accretions which Herter ('08) calculated from average body composition. He estimated that children should store an average of 0.36 meq. of potassium, 0.29 meq. of sodium and 0.25 meq. of chlorine daily. If his calculations were correct, the children were storing adequate amounts of these minerals to produce normal growth.

The children, as previously reported (Hawks, Bray and Dye, '38), gained weight during the entire experiment, but they gained from 2 to 6 times as fast on the 4- as on the 3-gm. protein diet. Being normal, they were undoubtedly producing both bone and soft tissue on both diets, but the composition of the growth was not identical. The higher nitrogen balances on the 4-gm. protein diet as well as the retention ratios between calcium, phosphorus and nitrogen (Hawks, Bray, Wilde and Dye, '42) indicated that the children produced a larger proportion of soft tissue than bone tissue on this diet than on the lower protein diet. The figures for potassium, sodium and chlorine substantiate the above conclusion as well as indicate that the soft tissue increase occurred both in muscle and extramuscle tissue.

Shohl ('39) and Peters and Van Slyke ('32), in reviewing the literature on this subject, say that potassuim is held within the tissue cell itself while sodium is found mainly in the interstitial fluids. Since the composition of the fluids remains exceptionally constant, it can be assumed that $(Na^+) + (K^+)$ when expressed in meq./kg. of water, represents the total amount of body fluid, and that the relationship between sodium and potassium indicates the proportion of extra- and

intracellular fluid. Thus on the basis of apparent sodium and potassium retentions, gains in body tissue can be expressed in terms of body fluids. Peters and Van Slyke, using the principle established by Gamble, Ross and Tisdall ('23), suggest the following formulae for calculating the liters of muscle and extramuscle fluid retained.

$$\frac{(Na^+) - 0.425 \ (K^+)}{148} = \text{liters of extramuscle fluid.}$$

$$\frac{(K^+) - 0.017 \ (Na^+)}{112} = \text{liters of muscle fluid.}$$

$(Na^+)$ in muscle fluid = 0.425 $(K^+)$. $(K^+)$ in extramuscle fluid = 0.017 $(Na^+)$.

$(Na^+)$ of extramuscle fluid = 148 meq./1. of water.

$(K^+)$ of muscle fluid = 112 meq./1. of water.

Calculations according to the above formulae (table 2) show that with the exception of child J, the cubic centimeters of fluid derived from both muscle and extramuscle sources increased on the high protein diet. Thus it can be said that the additional weight gains of the children on the higher protein diet were composed of both muscle and extramuscle tissue.

Both types of tissue, however, did not increase in the same proportion in the two experiments. In the first, muscle fluid

TABLE 2

*Muscle and extramuscle fluid stored per kilogram of body weight.*

| EXPERI- MENT | CHILD | PROTEIN IN DIET | MUSCLE FLUID | | EXTRAMUSCLE FLUID | | RATIO MUSCLE/ EXTRA- MUSCLE FLUID |
|---|---|---|---|---|---|---|---|
| | | | Amount | Increase with dietary protein | Amount | Increase with dietary protein | |
| | | gm. | cc. | % | cc. | % | |
| I | B | 3 | 2.29 | | 0.81 | | 2.83/1 |
| | | 4 | 2.73 | 19.2 | 0.93 | 14.8 | 2.93/1 |
| | D | 3 | 1.31 | | 0.72 | | 1.82/1 |
| | | 4 | 2.73 | 108.4 | 0.80 | 11.1 | 3.41/1 |
| II | V | 3 | 2.72 | | 1.20 | | 2.27/1 |
| | | 4 | 3.16 | 16.2 | 1.53 | 27.5 | 2.07/1 |
| | C | 3 | 2.72 | | 1.14 | | 2.39/1 |
| | | 4 | 2.89 | 6.3 | 1.28 | 12.3 | 2.26/1 |
| | J | 3 | 2.47 | | 0.75 | | 3.29/1 |
| | | 4 | 2.28 | —7.7 | 1.15 | 53.3 | 1.98/1 |

increased 19.2 and 108.4%, while extramuscle fluid increased only 14.8 and 11.1% for subjects B and D, respectively. In the second experiment, the reverse conditions were true. The percentage increases in muscle fluid on the higher protein diet were less than the percentage increases in extramuscle fluid. The ratios of the actual amounts of muscle to extramuscle fluid stored also show this same relationship. On the 4-gm. protein diet these ratios increased in the first, and decreased in the second, experiment. The fact that mineral intakes were slightly higher on the higher protein diet in the first experiment, and practically the same on the two diets in the second experiment, suggests that protein alone without the simultaneous addition of minerals cannot produce extra protein tissue as effectively as additional protein plus minerals. Thus potassium may be a limiting factor in the growth of muscle tissue. On the other hand, it is possible that the slight increase of 4.5 calories per kilogram on the 4-gm. protein diet in the first experiment may have had a protein sparing action and caused the increase in protein tissue.

Recent evidence indicates that it is questionable whether there is any chlorine in muscle cells, all of it being found in the extracellular fluids. Therefore the large increases in chlorine retentions on the higher protein diet lend further evidence to the conclusion that this diet caused an increase in extramuscle or extracellular fluid. Further, the greater proportional increase in chlorine retention for the children in the second, as compared to those in the first experiment, substantiated the conclusion that they produced a larger proportion of extracellular fluid on the 4- than on the 3-gm. protein diet.

Sodium and chlorine are so closely associated both within and without the body that it is often assumed that their utilization is parallel. They may, however, perform quite different and specific functions in the body. In this experiment, sodium and chlorine utilizations were not entirely parallel. The diet contained more chlorine than sodium and had no large excess of added salt. Fecal outputs for both minerals were all below 0.1 meq./kg., but chlorine values were slightly higher than

those for sodium. Urinary excretions of chlorine were also greater than those for sodium and followed intake figures to some extent, although the proportion of the intake excreted was lower on the 4- than on the 3-gm. protein diet. The relationships between sodium and chlorine retentions indicated that the children were not all producing the same type of extracellular tissue. The two boys in the first experiment and subject J stored more chlorine than sodium during the 4-gm. protein diet. All three children in the second experiment stored more sodium than chlorine on the 3-gm. protein diet, and subjects V and C stored equal amounts of the two minerals on the 4 gm. protein diet. The high chlorine retentions suggest that the children may have been producing some particular type of tissue, as blood cells, which contain large amounts of chlorine or that they may have been storing chlorine along with water in the skin or subcutaneous tissue.

## SUMMARY

1. Five pre-school children received constant diets containing first 3 and then 4 gm. of protein per kilogram of body weight.

2. The amounts of potassium, sodium and chlorine which these children stored on both diets were similar to estimated daily accretions and to values which other investigators have obtained for children of similar age.

3. The 4-gm. protein diet produced little, if any, change in potassium retentions for some of the children, but caused a slight increase in others. This diet resulted in slightly higher sodium retentions and distinctly higher chlorine retentions for all of the children.

4. On the basis of potassium and sodium retentions, the percentage increase in muscle fluid on the higher protein diet in the first experiment was greater than the percentage increase in extramuscle fluid, while in the second experiment, there was a smaller percentage increase in muscle than in extramuscle fluid. At the same time the minerals in the diet increased slightly in the first, and remained the same in the

second, experiment. These facts suggest that an increase in protein without an increase in minerals did not cause as much muscle production as an increase in both protein and minerals and that potassium, since it is primarily found in muscle tissue, may be a limiting factor in muscle growth.

5. The increased chlorine retentions on the higher protein diets suggested that specific types of tissue may have been produced at that time.

### LITERATURE CITED

BARBER, H. H., AND I. M. KOLTHOFF 1928 A specific reagent for the rapid gravimetric determination of sodium. J. Am. Chem. Soc., vol. 50, p. 1625.

———————— 1929 Gravimetric determination of sodium by uranyl zinc acetate method. II. Application in the presence of rubidium, calcium, potassium, lithium, phosphate and arsenate. J. Am. Chem. Soc., vol. 51, p. 3233.

BUTLER, A. M., AND E. TUTHILL 1931 An application of the uranyl zinc acetate method for the determination of sodium in biological material. J. Biol. Chem., vol. 93, p. 171.

GAMBLE, J. L., G. S. ROSS AND F. F. TISDALL 1923 The metabolism of fixed base during fasting. J. Biol. Chem., vol. 57, p. 633.

HAWKS, J. E., M. DYE AND M. M. BRAY 1937 An improved technic for metabolism studies in pre-school children with a statistical determination of its reliability. J. Nutrition, vol. 13, p. 51.

———————— 1938 The influence of diet on the nitrogen balances of pre-school children. J. Nutrition, vol. 15, p. 125.

HAWKS, J. E., J. M. VOORHEES, M. M. BRAY AND M. DYE 1940 The influence of the nitrogen content of the diet on the calorie balances of pre-school children. J. Nutrition, vol. 19, p. 77.

HAWKS, J. E., M. M. BRAY, M. O. WILDE AND M. DYE 1942 The interrelationship of calcium, phosphorus and nitrogen in the metabolism of pre-school children. J. Nutrition, vol. 24, p. 283.

HERTER, C. A. 1908 On infantilism from chronic intestinal infection. The Macmillan Company, New York.

HUMMEL, F. C., H. A. HUNSCHER AND I. G. MACY 1939 Effect of irradiated milks on storage of nitrogen and acid-base minerals in children. Am. J. Dis. Child., vol. 58, p. 753.

HUNSCHER, H. A., F. C. HUMMEL AND I. G. MACY 1940 Effect of varied banana intake on nitrogen and mineral balances of normal children. Am. J. Dis. Child., vol. 60, p. 509.

PETERS, J. P., AND D. D. VAN SLYKE 1932 Quantitative Clinical Chemistry, vol. I and II. The Williams and Wilkins Company, Baltimore, Maryland.

SHOHL, A. T. 1923 Mineral metabolism in relation to acid-base equilibrium. Physiol. Rev., vol. 3, p. 509.

———— 1939 Mineral metabolism. Am. Chem. Soc., Monograph Series No. 82. Reinhold Publishing Corporation, New York.

SOUDERS, H. J., H. A. HUNSCHER, F. C. HUMMEL AND I. G. MACY 1939 Influence of fluid and of evaporated milk on mineral and nitrogen metabolism of growing children. Am. J. Dis. Child., vol. 58, p. 529.

STEARNS, G. 1939 The mineral metabolism of normal infants. Physiol. Rev., vol. 19, p. 415.

SWANSON, W. W., AND L. V. IOB 1933 Loss of minerals through the skin of infants. Am. J. Dis. Child , vol. 45, p. 1036.

# THE RIBOFLAVIN REQUIREMENT OF THE DOG [1]

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TWO FIGURES

It has been demonstrated that the riboflavin requirement of the rat is increased markedly on a high fat diet (Mannering et al., '41). The present study was undertaken to determine whether the riboflavin requirement of the growing dog is influenced in a similar manner by the fat content of the diet. To this end, the riboflavin requirement of the growing dog, expressed in terms of micrograms of riboflavin per kilogram of body weight per day, was determined both on a high carbohydrate and high fat diet.

The recent recognition that choline is essential in the dietary of the dog (Schaefer et al., '41) has made it desirable to reinvestigate the symptoms of riboflavin deficiency as observed on highly purified diets containing ample amounts of choline. This is of special interest since the symptoms and pathology of riboflavin deficiency in the dog have been described by previous workers who have employed diets in which known amounts of choline were not supplied.

## METHODS

*Care of dogs.* Mongrel pups which had been given a vermifuge were placed on the experimental diet at 6–7 weeks of age.

The pups were maintained on a milk diet from weaning until the time the experiment began. Fresh ration was supplied ad libitum. The daily food consumption of each dog was recorded and the animals were weighed daily. The dogs within the two groups employed were litter mates.

*Rations employed.* The high carbohydrate ration used is essentially that of Street and Cowgill ('39) and has the following percentage composition: sucrose, 66; casein (acid washed), 19; cottonseed oil, 8; cod liver oil, 3; and salts III (McKibbin et al., '39), 4.

As supplements the following crystalline vitamins [3] were fed: thiamine, pyridoxine and pantothenic acid at 100, 60 and 200 µg. per kilogram body weight per day, respectively; nicotinic acid and choline at 2 and 25 mg. per kilogram per day, respectively. The vitamins were prepared in a single solution of 20% ethanol at such a concentration that the solution could be fed at the rate of 1 cc. per kilogram of body weight on alternate days. As a source of the remaining filtrate factors required by the dog a butanol extract (Axelrod et al., '41) of liver powder [4] was fed in the ration at a level equivalent to 3% of the original liver powder. The riboflavin content of this preparation was less than 0.2 µg. per gram of liver powder as determined by a microbiological technique (Snell and Strong, '39). Riboflavin was fed on alternate days in a solution containing 250 µg. per cubic centimeter. The casein employed in this ration contained approximately 1 µg. of riboflavin per gram. Since the dogs consumed an average of 45 gm. of ration per kilogram of body weight per day, the daily intake of riboflavin supplied by the casein was 8.6 µg. per kilogram of body weight.

The high fat diet was prepared by isocalorically replacing a portion of the carbohydrate of the low fat ration with lard. The actual procedure was to replace 36 parts of sucrose with 16 parts of lard. To increase the consistency and palatability

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[3] We are indebted to Merck and Co., Rahway, N. J., for generous supplies of the vitamins employed in this study.

[4] The liver concentrate powder (1–20) was supplied by the Wilson Laboratories.

of the ration the 8 parts of cottonseed oil were replaced by 8 parts of lard. The high fat ration had the following composition: sucrose, 30 parts; lard, 24; casein (acid washed), 19; salts III, 4; and cod liver oil, 3. The ration was prepared every few days and stored in the cold room. Under these conditions the ration was easy to handle and was very acceptable to the dogs. During the feeding of the high fat ration the thiamine supplement was fed at a level of 60 µg. per kilogram of body weight per day and the choline supplement at a level of 50 mg. per kilogram of body weight per day. The other experimental conditions remained as previously described.

*Urinary riboflavin determinations.* The dogs were placed in wire-bottom metabolism cages and the 24-hour urine samples were collected under toluene in dark bottles. Before storing in the refrigerator previous to assay the pH of the urine was adjusted to 6.8. The riboflavin content of the urine was determined by a microbiological method (Snell and Strong, '39).

*Other determinations.* The fat content of the liver was determined in the following manner. The freshly extirpated liver was oven-dried at 80°C. and the total lipids were subsequently extracted with chloroform (Channon et al., '37) in a continuous extractor for 24 hours. Blood was obtained by venepuncture and hemoglobin was determined colorimetrically by the method of Evelyn ('36).

<div align="center">EXPERIMENTAL</div>

### A. High carbohydrate ration

Dogs I and II received 60, and dogs III and IV received 100 µg. of riboflavin per kilogram of body weight, respectively.

*Dogs I and IV.* After 47 days the riboflavin supplement was withheld. Dog I began immediately to decline in weight while dog IV continued to grow at the usual rate for 12 days and then reached a weight plateau (fig. 1). After 16 days · without riboflavin, the dogs were given the supplement as before. In both cases a rapid growth response was observed.

Twenty-eight days later (91 days on experiment) the ribo-
flavin supplements were again withdrawn. It will be noted
from the figure that dog I continued to grow for 4 days and
then began to decline rapidly in weight. On the other hand,
dog IV continued to grow for some time and only after a
26-day period without riboflavin supplementation did the dog
begin to lose weight. Dog I died 44 days after the last ribo-
flavin supplement was given. The typical riboflavin deficiency
symptoms were observed. Dog IV was still in good condi-
tion at this time.
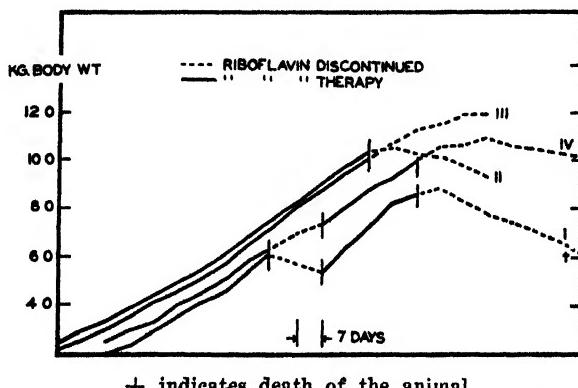


+ indicates death of the animal.

Fig. 1  Growth of dogs on a high carbohydrate ration. Dogs I and II received
60, and dogs III and IV received 100 µg. of riboflavin per kilogram of body
weight per day, respectively, during the periods indicated.

*Dogs II and III.* A good rate of growth was observed in
both dogs for a period of 91 days (fig. 1). At this time the
riboflavin supplement was discontinued. Dog II attained a
stationary weight within 10 days and then began to lose weight
rapidly. In contrast, dog III, receiving the 100 µg. supplement,
continued to grow for 22 days. At this time the weight reached
a constant level and no weight loss was observed even after
31 days without riboflavin.

The growth rates of all the animals in this group were
similar and averaged 525 gm. per week during the period when
riboflavin was being administered.

## B. High fat ration

Four dogs were placed on the high fat diet and given supplements as follows: Dog V received no supplement, dog VI received 50 µg. of riboflavin per kilogram of body weight per day, while dogs VII and VIII received 100 µg. of riboflavin per kilogram of body weight per day.

*Dog V.* Although this animal received no daily riboflavin supplementation, a test dose of 1.35 mg. of riboflavin was injected subcutaneously after 18 days on experiment, and 1 mg.
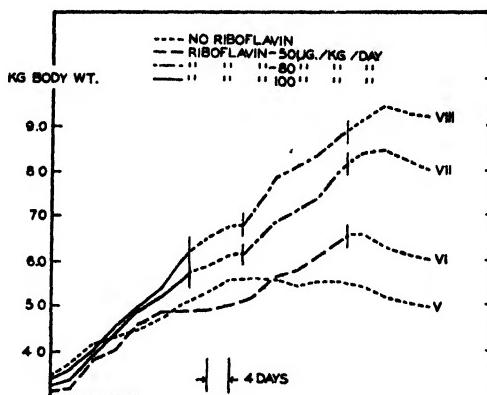


Fig. 2 Growth of dogs receiving a high fat diet supplemented with varying amounts of riboflavin.

of riboflavin was given orally by mistake on the twenty-fourth day. The dog grew fairly well for 32 days (fig. 2) and then reached a weight plateau. The weight remained constant over a considerable period of time and eventually the dog lost weight and died. This animal was observed to eat its feces and it is possible that this helped to maintain the dog for some time (Street et al., '41).

*Dog VI.* This dog received 50 µg. of riboflavin per kilogram of body weight per day for 53 days. Growth was maintained at a moderate rate until, after 53 days on experiment, the riboflavin supplement was withdrawn (fig. 2). At this time it will be noted that the weight of dog VI was about a kilogram

more than that of dog V but nearly 2 kg. less than the average weight of dogs VII and VIII receiving 100 µg. of riboflavin per kilogram of body weight per day. Growth ceased soon after the removal of the riboflavin supplement and a slow decline in weight ensued.

*Dogs VII and VIII.* These dogs received 100 µg. of riboflavin per kilogram of body weight per day for 25 days. At this time riboflavin was withheld and the animals were allowed to continue without supplement for 10 days until they appeared to reach a weight plateau (fig. 2). The dogs were then given a supplement of 80 µg. of riboflavin per kilogram of body weight per day and an immediate growth response was noted. After 18 days on this regimen the riboflavin supplement was discontinued. Growth soon ceased and both dogs maintained their weight for a short period of time before any appreciable weight losses were observed.

For comparison purposes the riboflavin intake for each dog in terms of food consumption has been calculated for the first 30 days on experiment and the values are given in table 1. The calculated figures expressing the riboflavin intake per gram of food consumed were obtained by dividing the total riboflavin intake during this period by the total food intake.

*Urinary riboflavin excretion.* The daily urinary riboflavin excretion was determined during the periods in which the dogs were receiving riboflavin supplements. The results appear in table 1.

*Symptoms of riboflavin deficiency.* After the completion of the riboflavin requirement studies, three of the dogs in each group were fed only their respective basal rations until the collapse syndrome appeared. Before the appearance of the collapse syndrome there elapsed intervals of time ranging from 6 to 13 weeks after the withdrawal of the riboflavin supplement. The fourth animal in each group was made an inanition control to one of the experimental mates. The control dog was given 100 µg. of riboflavin per kilogram of body weight per day and its food intake was restricted to that of its paired mate. The following observations are reported:

1. Fatty livers. All dogs on the basal diets showed typical friable, fatty, "yellow" livers upon autopsy. The fat content of the fatty livers ranged from 42.4 to 55.5% on the moisture-free basis. The livers of the two control dogs were normal in color and texture and contained 13.1 and 16.5% fat, respectively.

TABLE 1

*Riboflavin intake and excretion.*

| DOG | DURATION OF EXPERIMENT | RIBOFLAVIN SUPPLEMENT | | RIBOFLAVIN EXCRETION [1] |
| | | Per kg. body weight per day | Per 100 gm of ration consumed | Per kg body weight per day |
|---|---|---|---|---|
| | *days* | *μg.* | *μg* | *μg* |
| I | 45 | 60 | 140 | 4.8 |
| II | 40 | 60 | 158 | 4.8 |
| | 85 | | | 6.4 |
| III | 40 | 100 | 246 | 6.4 |
| | 85 | | | 47.0 |
| IV | 45 | 100 | 280 | 7.0 |
| V | 99 | 0 | (—) [2] | 1.5 |
| VI | 43 | 50 | 185 | 4.7 |
| VII | 43 | 100–80 | 244 | 5.6 |
| VIII | 50 | 100–80 | 254 | 6.5 |

[1] Each figure is an average of 3 or more values.

[2] Received test dose of 1.35 mg. subcutaneously after 18 days on experiment, and 1 mg. orally by mistake on 24th day. Was a coprophagist.

2. Anemia. The hemoglobin values varied from 9.9 to 12.3 gm. per cent. There was no correlation between the degree of riboflavin deficiency and the hemoglobin concentrations.

3. Muscular weakness. A muscular weakness of the hind quarters was observed in all of the deficient animals shortly before the appearance of the collapse syndrome. It did not appear to be due to inanition since neither of the control dogs was similarly affected.

4. Dermatitis. Five of the six basal dogs exhibited a dry, flaky dermatitis usually accompanied by a marked erythema on the hind legs, chest and abdomen.

5. Eye symptoms. In the dogs deprived of riboflavin, eye symptoms appeared in 4 to 9 weeks. A watery or purulent discharge from the eye accompanied by a conjunctivitis was first manifested. A few days later a vascularization of the cornea became noticeable and at the same time the nictitating membranes were pulled forward and the eyeball rotated dorsally in the manner described by Street et al. ('41). Three of the deficient dogs developed opacities of the cornea. The condition of dog I after 51 days on the basal ration was described by Dr. Mark E. Nesbit [5] as follows: "Bilateral conjunctivitis. Right eye, generalized grayness of the cornea with one area, round in shape, much more involved. Left cornea has some central grayness of the epithelial layer but otherwise clear. With the ophthalmoscope no blood vessels could be detected." No eye symptoms were observed in the control dogs.

6. Heart rate. In five of the deficient dogs heart rates of 140 to 190 beats per minute were observed a few hours before the collapse syndrome appeared.

### DISCUSSION

From the results with the dogs on the high carbohydrate diet (fig. 1) it seems permissible to conclude that while there was no difference in the growth rates on the two levels of riboflavin employed (60 and 100 μg. per kilogram per day) there was a marked difference in the degree of riboflavin storage. When the high fat diet was employed, both 80 and 100 μg. of riboflavin per kilogram per day supported excellent growth. In one dog, the 50-μg. supplement did not prove to be sufficient for optimal growth. With either ration the riboflavin requirement for the growing dog can be tentatively placed between 60 and 100 μg. per kilogram of body weight per day. It should be pointed out that the ration is not completely riboflavin-free but that it actually contributes approximately 10 μg. of riboflavin per kilogram of body weight daily. Street and Cowgill

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('39) have suggested 25 µg. of riboflavin per kilogram of body weight per day as the riboflavin requirement for the adult dog. This value has been questioned since the food intake of the animals was restricted and the dogs lost weight during the experimental period (Axelrod et al., '40). Axelrod et al. ('41) have expressed the minimal riboflavin requirement of the growing dog as 200 µg. per 100 gm. of ration. In their work, 100 µg. of riboflavin per 100 gm. of ration proved to be inadequate while 400 µg. per cent was ample. These values are in fair agreement with the results of the present study. It is evident from table 1 that 50 or 60 µg. of riboflavin per kilogram of body weight per day corresponds to less than 200 µg. per cent while 80 to 100 µg. per kilogram per day amounts to more than 200 µg. per cent of riboflavin. Hughes ('40) has stated that the riboflavin requirement of the pig is between 1 and 3 mg. per 100 lbs. of body weight per day. Sebrell et al. ('41) have concluded, on the basis of riboflavin excretion studies, that the human riboflavin requirement is more than 35 and somewhat less than 60 µg. per kilogram per day. An estimate of the riboflavin requirement of the human adult can be obtained from our data with the following considerations. The function of riboflavin is related to its ability to serve as an integral component of a number of enzyme systems involved in the metabolism of carbohydrates, proteins and, possibly, fat. Thus, the need of the organism for riboflavin could be conditioned by the extent of the energy requirement. It is recognized that the energy requirement of a growing dog per kilogram of body weight is approximately twice that of an adult dog of the same weight. If the riboflavin requirement is in direct proportion to the energy requirement, it follows from our data that the riboflavin requirement is approximately 40 µg. per kilogram for an adult dog. However, a further correction is required since the food maintenance requirements of the adult dog vary with the weight of the dog. Our dogs weighed approximately 6 kg. and it can be noted from the maintenance prediction values of Brody, Proctor and Ashworth ('34) that the caloric requirement per kilogram

of a 50-kg. dog is 49/87 that of a 6-kg. dog. Again, presupposing a direct relationship between energy and riboflavin requirement, it follows that the riboflavin requirement of a 50-kg. dog would, therefore approximate 49/87 × 40 or 23 µg. per kilogram of body weight. On this basis the minimum riboflavin requirement of an adult human weighing 70 kg. would lie in the range of 1.6 mg. per day.

In general, it may be concluded that the riboflavin requirement of the growing dog fed the ration employed in the present study is not a function of the fat content of the ration. These results appear to be at variance with those previously reported for the rat (Mannering et al., '41). Recent work, however, may offer an explanation for this apparent difference between species. Mannering and Elvehjem ('42) have noted that the influence of fat on the dietary requirement of the rat for riboflavin is observed only when dextrin is employed as the source of carbohydrate; with sucrose as the carbohydrate component, no effect of fat was noted. It remains to be determined whether the substitution of dextrin for sucrose in the dog rations would make it possible to observe the effect of fat upon the riboflavin requirement.

It is of interest to note that the urinary riboflavin excretion of the dogs in the present study, during the period of riboflavin supplementation and rapid growth, is very similar to that previously observed (Axelrod et al., '41) in dogs maintained on a riboflavin deficient ration and receiving no riboflavin therapy. These values indicate a fairly complete utilization of the administered riboflavin during the period of rapid growth. The urinary excretion data lend further support to the conclusion that 60 µg. of riboflavin per kilogram of body weight per day is a minimal level. Thus, dog II, receiving this level of riboflavin therapy, showed only a small increase in the urinary riboflavin excretion per kilogram of body weight with the approach of maturity, while dog III, receiving 100 µg. of riboflavin per kilogram of body weight per day, showed a sevenfold increase in the urinary riboflavin excretion per kilogram of body weight (table 1).

The symptoms of riboflavin deficiency observed in the present study where known amounts of choline were added to the ration did not differ from those observed by previous workers. Thus, choline does not appear to have been a limiting factor in rations previously employed for the study of riboflavin deficiency in the dog. The "yellow," fatty livers previously associated with riboflavin deficiency by Sebrell and Onstott ('38) were also observed in the present experiments. No fatty livers were observed in the inanition control dogs, an observation which is not in agreement with that reported by Street et al. ('41). The development of ocular symptoms observed in the present experiments is similar to those previously reported in the riboflavin deficiency of other species.

Sebrell and Onstott ('38) have described a bradycardia associated with the collapse syndrome of riboflavin deficiency. In the present experiment the heart rates observed at the time of collapse were, with one exception, between 140 and 190 beats per minute. Street and Cowgill ('39) have stated that the heart rate was normal or rapid during the period of collapse syndrome.

### SUMMARY

1. Under the experimental conditions described, the riboflavin requirement for the growing dog lies between 60 and 100 µg. per kilogram of body weight per day.

2. The isocaloric substitution of lard for sucrose does not increase the riboflavin requirement of the growing dog.

3. The following symptoms are associated with riboflavin deficiency: (1) fatty liver, (2) dermatitis, (3) muscular weakness of the hind quarters, (4) conjunctivitis, vascularization of the cornea, corneal opacities, and (5) tachycardia.

### LITERATURE CITED

AXELROD, A. E., M. A. LIPTON AND C. A. ELVEHJEM 1940 The production of uncomplicated riboflavin deficiency in the dog. Am. J. Physiol., vol. 128, p. 703.

———— 1941 Riboflavin deficiency in the dog. Am. J. Physiol., vol. 133, p. 555.

BRODY, S., R. C. PROCTOR AND W. S. ASHWORTH  1934  Basal metabolism, endo-
    genous nitrogen, creatinine and neutral sulfur excretions as functions
    of body weight. Univ. Mo. Res. Bul. 220.
CHANNON, H. J., A. P. PLATT AND J. A. B. SMITH  1937  The dietary prevention
    of fatty livers. Two analogues of choline. Biochem. J., vol. 31, p. 1736.
EVELYN, K. A.  1936  A stabilized photoelectric colorimeter with light filters.
    J. Biol. Chem., vol. 115, p. 63.
HUGHES, E. H.  1940  The minimum requirement of riboflavin for the growing
    pig. J. Nutrition, vol. 20, p. 233.
MANNERING, G. J., M. A. LIPTON AND C. A. ELVEHJEM  1941  Relation of dietary
    fat to riboflavin requirement of growing rats. Proc. Soc. Exp. Biol.
    and Med., vol. 46, p. 100.
MANNERING, G. J., AND C. A. ELVEHJEM  (Personal communication.)
McKIBBIN, J. M., R. J. MADDEN, SIMON BLACK AND C. A. ELVEHJEM  1939  The
    importance of vitamin B₆ and factor W in the nutrition of dogs. Am.
    J. Physiol., vol. 128, p. 102.
SCHAEFER, A. E., J. M. McKIBBIN AND C. A. ELVEHJEM  1941  Importance of
    choline in synthetic rations for dogs. Proc. Soc. Exp. Biol. and Med.
    vol. 47, p. 365.
SEBRELL, W. H., AND R. H. ONSTOTT  1938  Riboflavin deficiency in dogs. Pub.
    Health Rep., vol. 53, p. 83.
SEBRELL, W. H., R. E. BUTLER, J. G. WOOLEY AND H. ISBELL  1941  Human
    riboflavin requirement estimated by urinary excretion of subjects on
    controlled intake. Pub. Health Rep., vol. 56, p. 510.
SNELL, E. E., AND F. M. STRONG  1939  A microbiological assay for riboflavin.
    Ind. Eng. Chem. (Anal. Ed.), vol. 11, p. 346.
STREET, H. R., AND G. R. COWGILL  1939  Acute riboflavin deficiency in the dog.
    Am. J. Physiol., vol. 125, p. 323.
STREET, H. R., G. R. COWGILL AND H. M. ZIMMERMAN  1941  Further observa-
    tions of riboflavin deficiency in the dog. J. Nutrition, vol. 22, p. 7.

# METABOLISM AND GROWTH RATE OF RATS [1]

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This report presents data on day-to-day metabolism (oxygen consumption) of albino rats from birth to the age of 4 months, during which time they go through a series of striking changes, apparently not previously reported in the literature.

## METHODS

Oxygen consumption was measured daily on each of three litters (seven, eight and nine per litter) from birth to weaning at 22 days.

Four males and four females, six of which were selected from the nine-rat litter and two from the eight-rat litter, were then measured individually from 24 to 121 days.

Beginning with the thirty-second day, fasting as well as non-fasting data were secured on the same eight rats, the fasts varying from 14 to 20 hours, depending on age. Between 32 and 45 days, only one male and one female were fasted at a time; thereafter all rats were fasted simultaneously.

Measurements were made in an 8-chamber Regnault-Reiset volumetric type apparatus similar in principle to the 4-chamber apparatus for fowls described by Winchester ('40). Windows in the chambers permitted the observation of activity, and as readings were taken every 10 minutes for more than 1 hour, observed activity could be eliminated from the

results. Measurements were made at 30°C. between November, 1941 and April, 1942. The rats were housed at approximately 27°C.

## RESULTS

The results are presented in figures 1 and 2 and table 1.

In figure 1, metabolism in terms of Calories per square meter of body surface, as given in table 1, is plotted against age
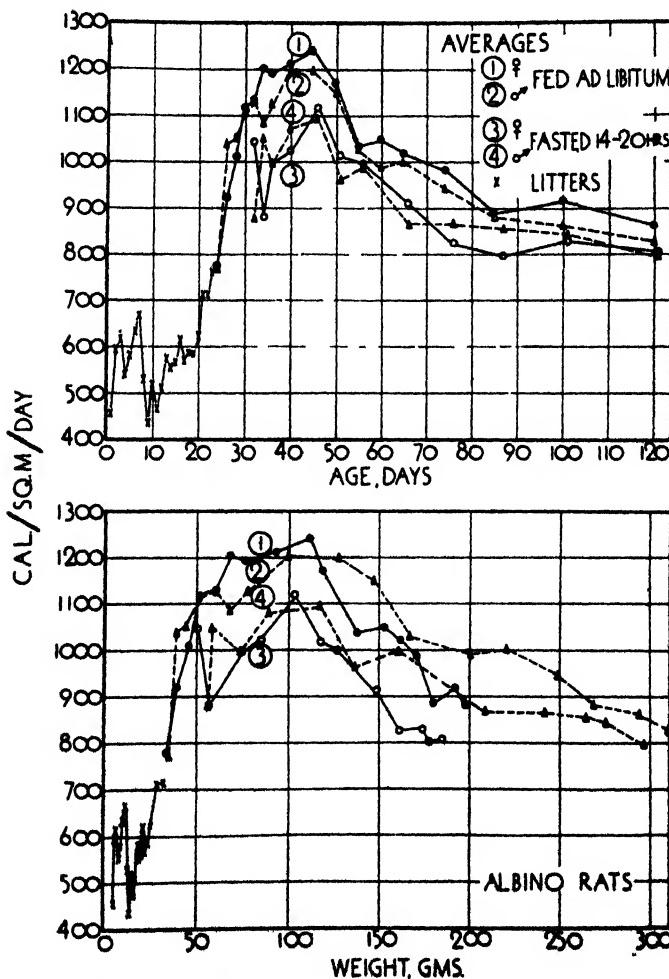


Fig. 1  Metabolism per unit surface area as a function of age (upper section) and of weight (lower section). Plotted from table 1.

TABLE 1

Growth and metabolism of albino rats.

(Basal values are shown on starred lines.)

| LITTERS | | | | FEMALES | | | | MALES | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Average per rat | | | | Average per rat | | | | Average per rat | | |
| Age | Body weight | Cal¹/Day | Cal/sq m²/Day | Age | Body weight | Cal¹/Day | Cal/sq m²/Day | Age | Body weight | Cal¹/Day | Cal/sq m²/Day |
| days | gm. | | | days | gm | | | days | gm | | |
| 1 | 5.64 | 1.51 | 456 | 24 | 34.0 | 7.9 | 779 | 24 | 35.5 | 8.0 | 771 |
| 2 | 6.21 | 2.09 | 596 | 26 | 39.6 | 10.3 | 921 | 26 | 39.1 | 11.4 | 1038 |
| 3 | 7.07 | 2.31 | 614 | 28 | 46.0 | 12.4 | 1010 | 28 | 44.7 | 12.6 | 1053 |
| 4 | 8.37 | 2.33 | 552 | 30 | 52.2 | 14.8 | 1117 | 30 | 51.7 | 15.1 | 1114 |
| 5 | 9.74 | 2.70 | 581 | 32 | 60.8 | 16.5 | 1130 | 32 | 60.4 | 16.4 | 1130 |
| 6 | 10.7 | 3.11 | 635 | *32 | 50.5 | 13.6 | 1045 | *32 | 56.7 | 12.3 | 878 |
| 7 | 12.2 | 3.48 | 666 | 34 | 68.8 | 19.4 | 1203 | 34 | 68.4 | 17.6 | 1084 |
| 8 | 13.3 | 2.98 | 531 | *34 | 57.2 | 12.4 | 880 | *34 | 58.7 | 15.0 | 1048 |
| 9 | 14.2 | 2.55 | 437 | 36 | 76.6 | 19.8 | 1193 | 36 | 78.1 | 19.1 | 1126 |
| 10 | 15.5 | 3.22 | 520 | *36 | 75.9 | 16.8 | 998 | *36 | 74.2 | 16.5 | 995 |
| 11 | 16.3 | 2.97 | 466 | 40 | 93.3 | 23.2 | 1210 | 40 | 101.0 | 24.2 | 1204 |
| 12 | 17.4 | 3.41 | 512 | *40 | 85.6 | 18.6 | 1022 | *40 | 89.5 | 20.1 | 1080 |
| 13 | 18.9 | 4.06 | 578 | 45 | 112 | 26.7 | 1241 | 45 | 128 | 28.0 | 1198 |
| 14 | 19.8 | 4.05 | 559 | *46 | 104 | 22.9 | 1119 | *46 | 118 | 24.3 | 1094 |
| 15 | 20.8 | 4.24 | 564 | 50 | 119 | 26.2 | 1171 | 50 | 147 | 29.3 | 1149 |
| 16 | 21.7 | 4.74 | 616 | *51 | 118 | 22.5 | 1015 | *51 | 137 | 23.4 | 962 |
| 17 | 22.9 | 4.52 | 569 | 55 | 138 | 25.1 | 1035 | 55 | 167 | 28.4 | 1029 |
| 18 | 23.6 | 4.73 | 586 | *56 | 127 | 23.2 | 998 | *56 | 161 | 26.8 | 991 |
| 19 | 24.4 | 4.83 | 584 | 60 | 153 | 27.4 | 1048 | 60 | 201 | 30.8 | 991 |
| 20 | 25.4 | 5.29 | 624 | 65 | 162 | 27.6 | 1021 | 65 | 221 | 33.1 | 1002 |
| 21 | 28.9 | 6.53 | 713 | *66 | 149 | 23.4 | 912 | *66 | 209 | 27.6 | 867 |
| 22 | 32.1 | 7.02 | 714 | 74 | 171 | 27.7 | 987 | 74 | 249 | 33.6 | 946 |
| | | | | *76 | 162 | 22.4 | 824 | *76 | 242 | 30.2 | 865 |
| | | | | 85 | 180 | 25.6 | 884 | 85 | 269 | 32.9 | 882 |
| | | | | *87 | 178 | 23.0 | 800 | *87 | 264 | 31.6 | 857 |
| | | | | 100 | 192 | 27.7 | 917 | 100 | 293 | 33.9 | 862 |
| | | | | *101 | 174 | 23.6 | 829 | *101 | 276 | 31.9 | 843 |
| | | | | 120 | 197 | 27.1 | 880 | 120 | 310 | 33.7 | 826 |
| | | | | *121 | 185 | 23.8 | 806 | *121 | 296 | 31.4 | 797 |

¹ The heat production was calculated on the assumption that 1 liter of oxygen had a heat equivalent of 4.7 Calories for the litters and 4.9 Calories for the older rats. A value of 4.7 Calories was also assumed for the fasted rats.

² Surface area was computed from the equation, surface area in square meters = 0.0011 (weight in gm.)$^{2/3}$ set up as a representative average relation from the surface area data of Carman and Mitchell ('26), Lee ('29) and Diack ('30).

in the upper section and against weight in the lower section. Prior to weaning, the curves appear irregular, varying around an average value of 576. Following weaning, maximum values of 1100 for fasting and 1200 for ad libitum feeding are reached at about the age of 45 days, or at a body weight of 100 gm. From 45 days to 4 months, the fasting values fall to 800, and the non-fasting values to about 850. The detailed characteristics of these curves have not been previously explored, apparently, although Mitchell and Carman ('26) reported higher basal values for rats between 32 and 40 days of age than for older rats up to 189 days, and other data in the literature bear on certain sections of the curves.

The rising and declining course of the age-curve of metabolism per square meter (upper section of fig. 1) is, in general, similar to that reported for other species; Riddle, Nussman and Benedict ('32) have reviewed the early literature. As our measurements were not continued beyond 4 months, they do not indicate whether there is a subsequent rise with increasing age as reported by Benedict and MacLeod ('29), or a continued fall with age as reported by Davis ('37). In dairy cattle (Brody, Kibler and Ragsdale, '41) the declining phase is less pronounced and is followed by a slight rise as the cattle reach a productive age.

It is perhaps significant that metabolism per square meter (fig. 1) rises during the period of rapid growth (during the age interval 24 to 45 days the body weights increase about 3.5 times), and declines as the growth rate declines (by the fourth month growth has practically stopped). Similar relations between metabolism per unit area and growth rate have been noted by Du Bois ('16) for children and by Riddle, Nussman and Benedict ('32) for young pigeons. Coleman and Du Bois ('15) also reported a basal metabolic level 16% above normal in adult typhoid fever patients in their second and third weeks of convalescence when they were rapidly regaining weight.

Sex differences in metabolism per unit area are small at all ages (upper section of fig. 1) but quite apparent for

given body weights above 100 gm. (lower section of fig. 1). The lower heat production of the females for given body weights is, perhaps, associated in part with their more rapid decline in growth rate. After growth has practically stopped, both sexes reach the same level.
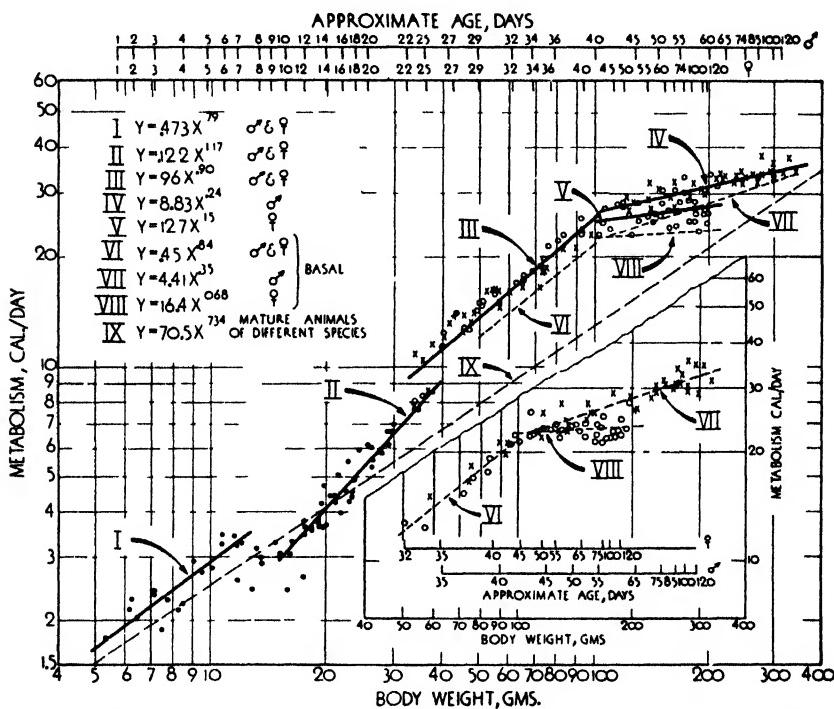


Fig 2   Metabolism as a function of body weight in albino rats (individual data plotted on logarithmic paper). Basal data are shown in the insert. The other data represent ad libitum feeding. The curves for the various phases of growth represent the equation $Y = ax^b$ fitted to the data by the method of least squares.

When total metabolism is plotted against body weight on logarithmic paper (fig. 2) the data appear to be distributed in four fairly distinct segments. To each of these segments the equation $Y = ax^b$ was fitted with results given in figure 2. The meaning of the first break, between segments I and II, is not clear, but unpublished data obtained by a different

method on different animals and at a different time substantiate its reality. The break between segments II and III coincides with weaning, but may be due to a change in method of measurement, from entire litters in a chamber with no control over activity to individual rats in a chamber with elimination of activity.

The final break, although it may not be as definite as pictured, seems to be real and associated with a change in percentage growth rate. Preceding the break (at about 100 gm. body weight) metabolism increases with the 0.8 to 0.9 power of body weight (a 1.0% increase in weight is associated with an 0.8 to 0.9% increase in metabolism); following the break, during the period of declining growth rate, metabolism increases with less than the 0.4 power of body weight (the values for the various curves are given in fig. 2). On a percentage basis then, total metabolism increases more rapidly with weight during the period of rapid growth rate than during the period of declining growth rate.

Sex differences in metabolism at a given body weight become apparent after 45 days and, perhaps, likewise may be attributed, in part, to differences in rates of growth, the males being chronologically younger (see age scale at top of fig. 2) and growing more rapidly than the females of the same body weight.

Curve IX (fig. 2) represents the relation of basal metabolism to body weight in mature animals of different species within the given weight range (Brody, Proctor and Ashworth, '34). During the period of rapid-percentage growth from weaning to 45 days, the basal metabolism (curve VI) of the rats is considerably higher than that of mature animals of different species, but as the rats near their maximum body weights their metabolism is very little higher than that of other species.

## SUMMARY

This paper presents day-to-day oxygen-consumption data on rats, from birth to 4 months, and brings out the following salient features:

The metabolic rate rises from about 550 Calories per square meter per day in early infancy (the low values perhaps being due to low muscle tonus and low endocrine activity) to 1100 basal or 1200 non-basal at the age of 45 days or at a body weight of 100 gm.; thereafter it declines to 800 basal and 850 non-basal, the decline in metabolic rate tending to parallel the decline in percentage growth rate. The decline in metabolic rate (as in growth rate) with increasing body weight is more rapid in the females than in the males.

When total metabolism is plotted against body weight on logarithmic paper, the resulting distribution exhibits "breaks" which apparently are associated, in part, with changes in percentage growth rate.

## LITERATURE CITED

BENEDICT, F. G., AND G. MacLEOD 1929 The heat production of the albino rat. II. Influence of environmental temperature, age, and sex; comparison with the basal metabolism of man. J. Nutrition, vol. 1, p. 367.

BRODY, SAMUEL, R. S. PROCTOR AND U. S. ASHWORTH 1934 Basal metabolism, endogenous nitrogen, creatinine and neutral sulphur excretions as functions of body weight. Missouri Agr. Exp. Sta. Res. Bul. 220.

BRODY, SAMUEL, H. H. KIBLER AND A. C. RAGSDALE 1941 Resting energy metabolism and ventilation rate in relation to body weight in growing Jersey cattle, with a comparison to basal energy metabolism in growing man. Missouri Agr. Exp. Sta. Res. Bul. 335.

CARMAN, G. G., AND H. H. MITCHELL 1926 Estimation of the surface area of the white rat. Am. J. Physiol., vol. 76, p. 380.

COLEMAN, W., AND E. F. DU BOIS 1915 Clinical calorimetry, seventh paper, Arch. Int. Med., vol. 15, p. 887.

DAVIS, J. E. 1937 The effect of advancing age on the oxygen consumption of rats. Am. J. Physiol., vol. 119, p. 28.

DIACK, S. L. 1930 The determination of the surface area of the white rat. J. Nutrition, vol. 3, p. 289.

DU BOIS, E. F. 1916 Clinical calorimetry, paper 12, Arch. Int. Med., vol. 17, p. 887.

LEE, M. O. 1929 Determination of the surface area of the white rat with its application to the expression of metabolic results. Am. J. Physiol., vol. 89, p. 24.

MITCHELL, H. H., AND G. G. CARMAN 1926 Effect of excessive amounts of vitamin B on the basal metabolism of rats. Am. J. Physiol., vol. 76, p. 385.

RIDDLE, O., T. C. NUSSMAN AND F. G. BENEDICT 1932 Metabolism during growth in a common pigeon. Am. J. Physiol., vol. 101, p. 251.

WINCHESTER, C. F. 1940 Seasonal metabolic and endocrine rhythms in the domestic fowl. Missouri Agr. Exp. Sta. Res. Bul. 315.

# EFFECTS OF PROLONGED DAILY TREATMENT OF NORMAL RATS WITH SALINE ANTERIOR PITUITARY EXTRACT

## I. SEXUAL DIFFERENCES IN APPETITE, GROWTH AND ORGAN WEIGHTS [1]

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ONE FIGURE

This investigation was undertaken for the purpose of elucidating the function of certain endocrine secretions regulating the distribution and disposition of nutrients and nutrient energy within the normal animal. Such information is especially necessary for an understanding of the utilization of dietary energy in normal nutrition. Toward this end observations have been made of daily injections of anterior pituitary extract (A.P.E.) as affecting appetite, growth, organ weights, protein metabolism and energy metabolism. Comparisons have been made of treated rats with untreated controls by paired and ad libitum feeding methods with both males and females as subjects.

This report is concerned specifically with the effects of the A.P.E. on appetite and growth, with emphasis on the different growth response of the sexes, and on the influence of appetite in this relation. The organ weights are considered,

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incidentally, as affording possible clues to the causes of the differences in growth.

Differences in growth response of male and female rats subjected to continuing injections of A.P.E. were studied extensively by Evans and Simpson ('31). They concluded that the differences between the sexes resulted from a difference in tissue reaction and were independent of gonads and growth rates. Lee and Schaffer ('34) demonstrated that the growth response of female rats to A.P.E. was independent of its appetite stimulating effect. Their results were confirmed by Marx, Simpson, Reinhardt and Evans ('42) by the use of a growth hormone preparation free from pituitary "target organ" hormones. Thus, the divergence of response of males and females to A.P.E. has been recognized, and its specific growth effect has been demonstrated with females, but no corresponding investigation with male rats has come to the attention of the authors.

## EXPERIMENTAL

The extract used in these investigations was prepared from the anterior lobes of beef pituitaries which were received frozen and kept in this condition until extracted. The extraction was carried out in a cold room as follows: 28 gm. of the glandular tissue was triturated with sand in a mortar surrounded by ice and stirred for $\frac{1}{2}$ hour with 200 ml. of a 1% solution of sodium chloride. The mixture was centrifuged, and the supernatant extract transferred in 10 ml. portions to individual test tubes for daily use. The extract was kept frozen until used. Fresh batches of the extract were prepared as needed. The daily injections, which were made intraperitoneally, consisted of 1 ml. of the extract representing 140 mg. of the fresh anterior pituitary tissue. Each milliliter of the extract contained 1.5 mg. of nitrogen.

The effects of daily injections of this extract during a period of 12–14 weeks were investigated, first in 1939, with normal young male rats, and later, in 1941, the experimental program was repeated, in its essential details, with female rats.

Injections were started when the rats were 24 days old and weighed about 50 gm.

The rats were kept in individual cages and all received a stock diet of a nutritively complete commercial animal food.[2] Food consumption and body weights were recorded daily. Tap water was allowed ad libitum and, for the male rats, the water consumption was measured. The A.P.E. had no effect on water consumption in the males, and its effect was not determined with females.

The rats were grouped in litter-mate triplets, five triplets each of males and females being used for the main experiments. Of each triplet, one rat served as the control and two were treated with the A.P.E. One of the treated rats was pair-fed with the control and the other was fed ad libitum. In the experiments with the male rats the control rat received no injection, but in the experiments with females the control rat was injected daily with 1 ml. of 1% sodium chloride solution.

During the course of the experiments with males, rats other than the subjects of the main experiment were used to check the potency of the A.P.E. Six rats (three controls, three treated), fed ad libitum, were started during the ninth week. Since the A.P.E. gave no indication of deterioration under the conditions of preservation used, and results were duplicated with several shipments of the fresh frozen glands, similar tests of potency were not made in the experiment with females.

In the third, sixth and twelfth weeks of the experiment with the paired male rats, and in the fourth, seventh and thirteenth weeks with the female rats, growth continuity was interrupted temporarily with fasting periods in which food was withheld for 1 or 2 days for the purpose of metabolism measurements. The first fasting test with the treated male rats fed ad libitum was made during the second week. Otherwise, the fasting measurements for the three rats of each triplet were made simultaneously.

[2] Purina dog chow.

After 13 to 14 weeks the rats were killed with illuminating gas, and the weights of the livers, kidneys, hearts, adrenals and thyroids were obtained. With the female rats, the weight of the pancreas was also obtained.[3]
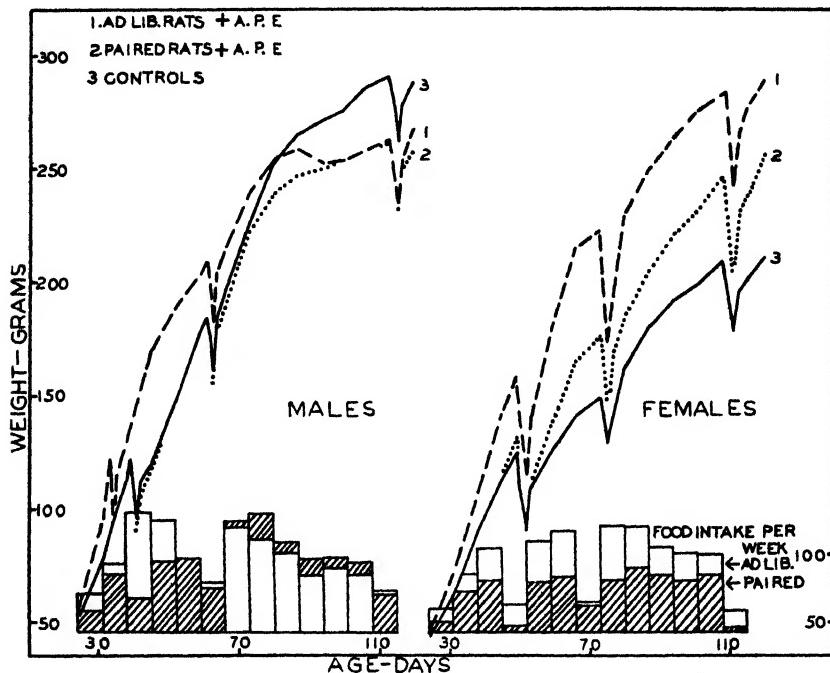


Fig. 1 Growth and food consumption of male and female rats as affected by daily injection of anterior pituitary extract. Breaks in growth curves represent fasting periods.

## RESULTS

### *Appetite and growth*

A notable difference in the response of the male and female rats to the A.P.E. was evident with respect to appetite and growth, especially after the treatment had been continued for several weeks. This difference between the sexes is conspicuous in the growth curves presented in figure 1, the breaks in the curves being caused by the fasting periods. The

[3] The authors are indebted to W. T. S. Thorp for dissecting out the organs.

essential features of the difference are illustrated by the data presented in table 1.

Both sexes responded to the A.P.E. initially with increased appetites, and the rats fed ad libitum outgrew the controls. The treated rats, which were pair-fed with the controls, were limited in food consumption by the latter, and their growth rates were essentially equal to those of the control rats for

TABLE 1

*Effect of prolonged daily injection of A.P.E. on the relationship of food intake to weight gained by rats.*

| PERIOD | FOOD INTAKE | | WEIGHT GAINED | | | FOOD FOR 1 GM. GAIN | | |
|---|---|---|---|---|---|---|---|---|
| | Control and pair-mate | Ad lib | Control | Pair-mate + A.P.E. | Ad lib. + A.P.E. | Control | Pair-mate + A.P.E | Ad lib. + A.P.E. |
| *Males* | | | | | | | | |
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm* | *gm* |
| Triplets | | | | | | | | |
| First 4 weeks | 309 | 423 | 95 | 93 | 135 | 3.3 | 3.3 | 3.1 |
| Second 4 weeks | 431 | 410 | 104 | 91 | 64 | 4.1 | 4.7 | 6.4 |
| Third 4 weeks | 398 | 364 | 34 | 21 | 6 | 11.7 | 19.0 | 60.1 |
| Accessory rats[1] | | | | | | | | |
| First 4 weeks | 375 | 463 | 130 | | 160 | 2.9 | | 2.9 |
| Second 4 weeks | 518 | 4°6 | 102 | | 74 | 5.1 | | 6.7 |
| *Females* | | | | | | | | |
| Triplets | | | | | | | | |
| First 4 weeks | 251 | 313 | 80 | 87 | 115 | 3.1 | 2.9 | 2.7 |
| Second 4 weeks | 306 | 414 | 38 | 53 | 71 | 8.1 | 5.8 | 5.8 |
| Third 4 weeks | 342 | 428 | 47 | 62 | 53 | 7.3 | 5.5 | 8.1 |

[1] Progress not interrupted by fasting periods.

the first 5 or 6 weeks. Following this period the responses of the males and females to the A.P.E. were distinctly different.

In the male rats no specific growth stimulation independent of food intake appeared at any time. The initial stimulating effect of the A.P.E. on appetite, which accounted for the extra gain in weight of the treated rats fed ad libitum, gradually disappeared, and during the final 7 weeks the appetites of the treated rats were depressed. It may be noted, in figure 1 and

table 1, that the food intake of the treated male rats fed ad libitum was actually less than that of the treated paired rats from the seventh to the twelfth weeks. Also, during this period three of the five treated paired rats consistently limited the food intake of their untreated control pair-mates.

Growth of the male rats receiving the A.P.E. was retarded during the same period in which appetite was depressed. These effects were partially independent, since the treated paired rats gained less weight than the controls after the seventh week. The treated male rats fed ad libitum lost their accumulated weight advantage in the ninth and tenth weeks, and completed the experiment on a weight par with the treated paired rats.

Male rats other than those of the main program, started under the same conditions 3 and 7 weeks later than the experiment proper, and not subjected to the fasting periods, also showed the reversal of response after the first 4 weeks. From the data in table 1 it may be noted that after the first 4 weeks all the treated male rats required more food per unit of gain in weight than did the controls. This difference was especially prominent in the last 4 weeks of the test, when the growth of the treated male rats fed ad libitum had practically ceased.

In contrast to the males, the female rats responded to the A.P.E. with increased appetites and growth, which effect continued to the end of the experiment. Partial indépendence of the appetite and growth-stimulating effects of the A.P.E. is evidenced after the fifth week by the extra weight gained by the treated rats which were pair-fed with the controls.

Some indication of a reactionary response by the female rats to the A.P.E. became evident in the last 4 weeks when the treated rats fed ad libitum gained less in weight than did the treated rats whose food intake had been restricted to that of the controls. In this period the food required per unit of gain in weight by the treated rats fed ad libitum was greater than that required by either the treated pair-fed rats or the controls. The appetite-stimulating effect of the A.P.E. in the treated rats fed ad libitum was still in evidence, but the

growth-promoting effect seemed to have waned during this period.

### Organ weights

The organ weights reported in table 2 were obtained by adjustment of the original data to a standard body weight approximating the mean body weight of the rats when killed.

TABLE 2

*The effect of prolonged daily injection of rats with A.P.E. on organ weights adjusted to standard body weight of 300 gm. by means of regression coefficients.*

| ORGAN | DURA-TION OF TREAT-MENT | REGRES-SION ON BODY WEIGHT | ORGAN WEIGHT | | SIGNIFI-CANCE OF DIFFER-ENCE [1] | REGRES-SION ON BODY WEIGHT | ORGAN WEIGHT | | SIGNIFI-CANCE OF DIFFER-ENCE |
|---|---|---|---|---|---|---|---|---|---|
| | | | Control | Treated | | | Control | Treated | |
| | | Males | | | | Females | | | |
| | *days* | *mg./gm.* | *gm.* | *gm* | | *mg./gm.* | *gm.* | *gm.* | |
| Liver | 100 | 52.5 | 12.7 | 11.3 | 140 | 43.6 | 14.5 | 12.9 | 400 |
| | 72 | | 14.3 | 11.5 | 90 | | | | |
| | 40 | | 14.3 | 12.4 | 40 | | | | |
| Kidneys | 100 | 4.5 | 2.51 | 2.46 | 2 | 4.5 | 2.14 | 2.12 | 2 |
| | 72 | | 2.69 | 2.38 | 40 | | | | |
| | 40 | | 2.68 | 2.48 | 8 | | | | |
| Heart | 100 | 2.1 | 0.90 | 0.92 | 1 | 3.1 | 1.01 | 0.96 | 20 |
| | 72 | | 0.98 | 0.95 | 1 | | | | |
| | 40 | | 0.98 | 1.02 | 1 | | | | |
| Adrenals | 100 | 0.136 | 0.036 | 0.044 | 45 | 0.294 | 0.078 | 0.074 | 6 |
| | 72 | | 0.038 | 0.050 | 18 | | | | |
| | 40 | | 0.039 | 0.053 | 17 | | | | |
| Thyroids | 100 | 0.045 | 0.011 | 0.018 | 58 | 0.024 | 0.017 | 0.017 | 1 |
| | 72 | | 0.017 | 0.020 | 350 | | | | |
| | 40 | | 0.018 | 0.016 | 1 | | | | |
| Pancreas | 100 | | | | | 1.2 | 0.94 | 1.09 | 21 |

[1] Numbers indicate odds against difference occurring by chance alone.

The adjustments were made by means of regression coefficients computed from all measurements with either sex regardless of treatment. The individual adjusted values were compared as paired variates by the use of Love's ('24) modification of Student's table for estimating the odds against the differences occurring by chance alone. Customarily, odds of twenty or

more are considered to indicate a probable significant difference, while odds of one hundred or more indicate a highly significant difference.

The results representing the 40 and 72 days' treatment in table 2 apply to the male rats other than those in the main experiment. These observations offer some indication as to the time of appearance of the changes which seem to be significant. The experimental groups treated during 100 days include the five triplets each of males and females.

The liver weights of the rats treated with the A.P.E. were significantly lower than those of the controls in both males and females. This difference was evident in the rats treated during 40 and 72 days, as well as in those treated during 100 days.

A probably significant enlargement of the adrenals was evident in the treated male rats, but no difference in the weight of these glands was noticeable in the female rats.

The thyroids of the treated male rats were larger than those of the controls, but this difference resulted from a decrease in weight of the control rat thyroids rather than from an enlargement of these glands in the treated rats. Apparently the thyroid weights in the control rats decreased with maturation, although within the group (rats of same age) the weights of the thyroids showed a positive correlation with body weight. No difference was evident between the thyroid weights of the treated females and the controls.

Other differences of possible significance may be noted in the lower kidney weights of the treated male rats at 72 days, and the lower heart weights and enlarged pancreas of the treated female rats.

### DISCUSSION

The results with the female rats corroborate, in a general way, those of Evans and Simpson ('31), Lee and Schaffer ('34) and Marx, Simpson, Reinhardt and Evans ('42). The results with the male rats differ from those of Evans and Simpson ('31) in that the rats of the latter did not show

the reversal of response encountered in the present investigation. Lee and Schaffer ('34) reported results with only two young male rats treated with A.P.E. during a period of 75 days. Neither of the two showed any significant growth stimulation above that recorded by their control pair-mates.

The depression of appetite and retardation of growth in the treated males were accompanied by a decrease in metabolic rate (results to be reported later). This would suggest either that thyroid activity was not related to the weights of the glands, or that the weight stasis of the thyroids in the treated rats resulted as a compensatory mechanism during the period in which the metabolic hormones of the A.P.E. were being counteracted. In this connection Turner and Cupps ('39) have suggested that the difference in growth rate of the sexes may be related to the difference in thyrotropic hormone production, since secretion of the thyrotropic factor increases during the period of most rapid growth in both sexes, but the female pituitaries contained only about half as much of the hormone as did those of the males.

Marx, Simpson and Evans ('42) reported that the thyrotropic hormone alone does not induce growth, but in association with the growth hormone it induces greater gains than does the growth hormone alone. Appetite effects were not recorded, so that the extent to which the extra gain resulted from increased intake or better utilization of food was not evident.

It is of interest that the liver weights in the treated rats of both sexes were significantly less than those in the controls. These results are in opposition to those of Lee and Freeman ('40), but are in apparent agreement with those of Marx, Simpson and Evans ('42) and Fraenkel-Conrat, Simpson and Evans ('42). According to the latter investigators a decreased liver weight is produced by a growth hormone, and an increased liver weight by thyroid stimulation. If this be true, the results reported herein would suggest that the effects of the growth hormone on liver weight and on growth were independent, since the growth responses of the males

and females were quite different, but the liver weight results were quite similar.

## SUMMARY

The effects on appetite, growth and organ weight of normal male and female rats treated daily during a period of 12 to 14 weeks with a 1% saline extract of bovine anterior pituitary lobes have been investigated by paired and ad libitum feeding.

In male rats there was no specific growth stimulation independent of food intake. An initial appetite-stimulating effect accounted for the extra gains in weight of treated male rats fed ad libitum. This effect gradually disappeared, and during the final 7 weeks the appetites of the treated male rats were depressed, and growth was retarded independently.

In female rats appetite and growth were stimulated independently. Treated females pair-fed with controls showed extra gain in body weight after the fifth week of treatment, while those fed ad libitum grew at about the same rate as normal untreated male rats.

The liver weights of the treated rats of both sexes were less than those of the controls, on the basis of equal body weight.

The weights of the adrenals and thyroids in the treated male rats were larger than those of the controls, but no difference in the weights of these glands was evident in the female rats. The effect on adrenals in the treated males appeared to be a true enlargement, but the difference in thyroid weight resulted from a decrease in the weight of these glands in the control rats rather than from an enlargement in the treated rats.

## LITERATURE CITED

Evans, H. M., and M. E. Simpson 1931 Hormones of the anterior hypophysis. Am. J. Physiol., vol. 98, p. 511.

Fraenkel-Conrat, H. L., M. E. Simpson and H. M. Evans 1942 Effect of purified pituitary preparations on liver weights of hypophysectomized rats. Am. J. Physiol., vol. 135, p. 398.

Lee, M. O., and N. K. Schaffer 1934 Anterior pituitary hormone and the composition of growth. J. Nutrition, vol. 7, p. 337.

LEE, M. O., AND W. FREEMAN  1940  Liver growth in rats treated with anterior pituitary growth hormone. Endocrin., vol. 26, p. 493.

LOVE, H. H.  1924  A modification of Student's table for use in interpreting experimental results. J. Am. Soc. Agron., vol. 16, p. 68.

MARX, W., M. E. SIMPSON, W. O. REINHARDT AND H. M. EVANS  1942  Response to growth hormone of hypophysectomized rats when restricted to food intake of controls. Am. J. Physiol., vol. 135, p. 614.

MARX, W., M. E. SIMPSON AND H. M. EVANS  1942  Synergism between thyrotropic and growth hormones of pituitary. Body weight increase in hypophysectomized rats. Proc. Soc. Exp. Biol. Med., vol. 49, p. 594.

TURNER, C. W., AND P. T. CUPPS  1939  The thyrotropic hormone of the pituitary of the albino rat during growth, pregnancy and lactation. Endocrin., vol. 24, p. 650.

# EFFECTS OF PROLONGED DAILY TREATMENT OF NORMAL RATS WITH SALINE ANTERIOR PITUITARY EXTRACT

## II. PROTEIN AND ENERGY METABOLISM [1]

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In the preceding paper (Voris, Kriss, Marcy and Bowman, '42) sexual differences in the responses of normal male and female rats to prolonged daily treatment with anterior pituitary extract (A.P.E.) were reported with respect to appetite, growth and organ weights. The present paper reports the results of measurements of the utilization of food protein, the katabolism of protein during fast and the energy metabolism of these rats as affected by the A.P.E.

### EXPERIMENTAL

The preparation of the saline A.P.E. and the general treatment of the rats have been described elsewhere (Voris et al., '42). Five litter-mate triplets of males and of females were used. Each triplet consisted of a control rat, its treated pairmate, and a treated rat fed ad libitum. Injections were started when the rats were 24 days old and weighed about 50 gm.

Fasting metabolism measurements were made with the paired male rats in the third, sixth and twelfth weeks, and with the female rats in the fourth, seventh, and thirteenth weeks of treatment. The first fasting test with the treated male rats fed ad libitum was made during the second week. With this exception, the fasting measurements for the three rats of

a triplet were made simultaneously, and all triplets were fasted during the same week.

Collection of the fasting urine for nitrogen determination and the measurements of the respiration metabolism were started about 24 hours after the last feeding. Urine collection was continued for 24 hours with the male rats and for 48 hours with the female rats.

For 10 days, during the fifth and sixth weeks of the experiment, the food intake was standardized at 14 gm. daily for the males, and at 12 gm. daily for the female rats. After 3 days on the equalized feeding treatment, collection of urine and feces was made for 7 days, at the end of which time the respiratory metabolism was measured.

In a separate experiment two fasting tests about 10 weeks apart and one feeding test were made with six pairs of male rats which were given only three daily injections with the A.P.E.

Respiration measurements were made by the Haldane open-circuit gravimetric technic with an apparatus similar to that described by Forbes, Kriss and Miller ('34), with modifications reported by Kriss ('38). The total $CO_2$ and $H_2O$ production and the insensible loss in weight of the rat during 7 hours' measurement were used for computation of the R.Q. The $CO_2$ production was determined hourly, but the $CO_2$ of the first hour, and any hour thereafter in which the activity of the rat was excessive, was excluded from the average. In the lighted chamber the rats were generally quiet, so the values determined for hourly heat production usually represented periods of 4 to 6 hours.

The $O_2$, $CO_2$ and heat involved in protein katabolism were computed from the urinary N, using the factors reported by Kriss and Voris ('37), i.e., 1 gm. of urinary N is equivalent to 5.96 liters $O_2$, 4.75 liters $CO_2$ and 26.71 Calories.

<center>RESULTS</center>

*Disposition of food nitrogen.* The results showing the effect of the A.P.E. on the disposition of food nitrogen are presented in table 1. The A.P.E. had no appreciable effect on the

TABLE 1

Urinary nitrogen excretion of fasting rats as affected by daily injections of anterior pituitary extract.

| GROUP | MALES | | | | | | FEMALES | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No of rats | Duration of treatment | Urinary N | Standard error of mean | Difference from control | Standard error of differences | No of rats | Duration of treatment | Urinary N | Standard error of mean | Difference from control | Standard error of differences |
| | | days | mg/day | ± | mg/day | + | | days | mg/day | ± | mg/day | ± |
| Controls | 5 | | 84 | 5 | | | 5 | | 100 | 5 | | |
| Pair-mates + A.P.E. | 4 | 15 | 128 | 10 | 44 | 11 | 5 | 25 | 139 | 16 | 59 | 16 |
| Ad lib. + A.P.E. | 2 | 10 | 90 | 12 | 6 | 13 | 4 | 25 | 141 | 9 | 41 | 10 |
| Controls | 5 | | 112 | 10 | | | 5 | | 104 | 5 | | |
| Pair-mates + A.P.E. | 5 | 38 | 184 | 10 | 72 | 14 | 5 | 47 | 141 | 8 | 37 | 10 |
| Ad lib. + A.P.E. | 4 | 38 | 189 | 12 | 77 | 16 | 4 | 45 | 162 | 3 | 58 | 6 |
| Controls | 5 | | 158 | 9 | | | 5 | | 124 | 2 | | |
| Pair-mates + A.P.E. | 5 | 88 | 175 | 16 | 17 | 18 | 5 | 86 | 167 | 9 | 43 | 10 |
| Ad lib. + A.P.E. | 4 | 88 | 222 | 15 | 64 | 18 | 4 | 86 | 155 | 9 | 31 | 9 |
| Control | 6 | | 147 | 18 | | | | | | | | |
| Pair mates + A.P.E. | 6 | 3 | 118 | 16 | —29 | 24 | | | | | | |
| Control | 6 | | 100 | 3 | | | | | | | | |
| Pair-mates + A.P.E. | 6 | 3 | 88 | 6 | —12 | | | | | | | |

apparent digestibility of food protein, as evidenced by the fecal nitrogen values which were essentially the same for the treated and the control rats of both sexes. Consequently the observed effects of the A.P.E. were referable entirely to the metabolism of the absorbed food nitrogen, as represented by the excretion of urinary nitrogen.

The female rats treated with the A.P.E. for 6 weeks retained over twice as much of the food nitrogen as did their control pair-mates with the same nitrogen intake. Male rats after 5 weeks of treatment retained 54%, and their control pair-mates 48% of the nitrogen absorbed. This difference was not statistically significant. However, male rats treated only three times with the A.P.E. showed a definite decrease in urinary N excretion. Thus, an initial food protein conservational effect of the A.P.E. appeared to have been somewhat counteracted by the sixth week of treatment in the male rats, but not in female rats. In our previous paper it was pointed out that the initial appetite-stimulating effect of the A.P.E. in male rats was being nullified at about this time.

*Urinary N excretion of fasting rats.* While the A.P.E. effected a conservation of food nitrogen by promoting its retention within the body, the rats treated with the extract for 2 weeks or more excreted considerably more N in the urine during fast than did the untreated controls (table 1). This effect was evident in all comparisons for both males and females in the three fasting periods. In only one comparison (paired male rats treated 88 days) is the difference of doubtful significance. On the other hand, with only three injections the average urinary N of the treated rats during fast was decreased. This was evident in both fasting tests made about 10 weeks apart on the same rats.

*Energy metabolism of food.* The effects of the A.P.E. on the energy metabolism of pair-fed rats nutritionally adjusted to constant food intake are shown in table 2.

The total heat production of male rats treated during 3 or 37 days, and female rats treated during 46 days, was increased by the A.P.E. However, in each case, the increase in total heat

## TABLE 2

*The disposition of food nitrogen, the respiratory quotients and heat production of pair-fed rats as affected by daily injections of anterior pituitary extract.*

| GROUP | DAYS TREATED | NITROGEN PER DAY | | | | R.Q. | | HOURLY HEAT | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Intake | Feces | Urine | Balance[1] | Total | Non-protein[1] | Total | Protein[1] | Non-protein[1] |
| | | mg. | mg. | mg | mg. | | | cal. | cal. | cal. |
| **Males** | | | | | | | | | | |
| Treated | 3 | 432 | | 128 ± 1 | | 0.97 | 1.00 | 1025 | 147 ± 1 | 878 ± 42 |
| Control | | 432 | | 168 ± 2 | | 0.99 | 1.05 | 878 | 183 ± 6 | 695 ± 21 |
| Difference | | | | −40 ± 2 | | | −0.05 ± 0.02 | 147 | −36 ± 7 | 183 ± 47 |
| Treated | 37 | 535 | 144 | 180 | 211 ± 16 | 0.99 | 1.03 | 1202 | 200 ± 17 | 1002 ± 46 |
| Control | | 535 | 142 | 205 | 188 ± 8 | 0.99 | 1.04 | 1122 | 229 ± 9 | 893 ± 33 |
| Difference | | | | | 23 ± 18 | | | 80 | −29 ± 19 | 109 ± 57 |
| **Females** | | | | | | | | | | |
| Treated | 46 | 451 | 129 | 172 | 150 ± 6 | 0.96 | 1.00 | 1088 | 192 ± 7 | 896 ± 17 |
| Control | | 451 | 133 | 247 | 71 ± 6 | 0.97 | 1.06 | 926 | 275 ± 6 | 651 ± 42 |
| Difference | | | | | 79 ± 8 | | −0.06 ± 0.016 | 162 | −83 ± 9 | 245 ± 46 |

[1] ± figures are standard errors of means or differences.

was the resultant of a diminished protein oxidation and a relatively larger increase in the oxidation of carbohydrate.

None of the non-protein R.Q. values was less than unity. The R.Q.'s for the male rats treated during 37 days were not appreciably different from those of the controls, and fat synthesis was indicated in both groups. In the treated female rats and male rats treated during 3 days the non-protein R.Q.'s indicated carbohydrate oxidation only, whereas in their controls fat synthesis was evident.

Computation of the glucose equivalents of the extra non-protein heat produced by the treated rats in these groups, and the fat synthesized by the control rats, indicated that the treated rats were oxidizing about twice as much extra carbohydrate as the control rats were synthesizing into fat.

The effect of the A.P.E. was noticeably greater in the male rats treated for 3 days than in those treated for 37 days, and the effect in the females was greater than in either of the male groups.

*Fasting energy metabolism.* The fasting energy metabolism data for male and female rats are presented in table 3. The effect of the A.P.E. on the basal metabolism of the rats seemed to parallel the appetite effect with respect to the difference in reaction observed between the sexes.

In the treated male rats the total heat production of fast was increased initially, but this stimulatory effect was later counteracted, and after the twelfth week of treatment the heat production was lower than that of the controls.

In the treated female rats the total heat production was greater than that of the controls throughout the 12 weeks of treatment, though the difference in the twelfth week was not as extensive as that observed in the fourth and the seventh weeks.

Between the sixth and the twelfth weeks of treatment in both sexes the relationship of the fasting heat of the treated rats fed ad libitum to that of the treated pair-fed rats was reversed. The basal metabolism of the former, which was initially higher, became lower than that of the latter group by the twelfth week of treatment.

## TABLE 3

*Fasting heat production of rats at different times during treatment with A.P.E.*

| GROUP | Males | | | | | | Females | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NO OF RATS | DAYS TREATED | WEIGHT | NON PROTEIN R.Q. | TOTAL HEAT | PROTEIN HEAT IN PER CENT OF TOTAL | NO OF RATS | DAYS TREATED | WEIGHT | NON PROTEIN R.Q. | TOTAL HEAT | PROTEIN HEAT IN PER CENT OF TOTAL |
| | | | *gm* | | *cal/hr* | | | | *gm* | | *cal/hr.* | |
| Control | 5 | 0 | 105 | 0.73 | 729 | 12.8 | 5 | 0 | 108 | 0.71 | 672 | 16.5 |
| Pair-fed + A.P.E. | 4 | 15 | 103 | 0.73 | 818 | 17.4 | 5 | 25 | 114 | 0.71 | 772 | 22.9 |
| Ad lib. + A.P.E. | 5 | 10 | 98 | 0.72 | 846 | 11.8 | 4 | 25 | 136 | 0.71 | 826 | 19.0 |
| Control | 5 | 0 | 176 | 0.73 | 898 | 13.9 | 5 | 0 | 147 | 0.71 | 776 | 14.9 |
| Pair-fed + A.P.E. | 5 | 38 | 171 | 0.73 | 886 | 23.1 | 5 | 47 | 173 | 0.71 | 943 | 16.6 |
| Ad lib. + A.P.E. | 5 | 38 | 200 | 0.72 | 949 | 22.1 | 4 | 45 | 203 | 0.71 | 997 | 18.1 |
| Control | 5 | 0 | 272 | 0.71 | 1039 | 16.9 | 5 | 0 | 194 | 0.72 | 807 | 17.1 |
| Pair-fed + A.P.E. | 5 | 88 | 246 | 0.72 | 941 | 20.7 | 5 | 86 | 229 | 0.71 | 947 | 19.6 |
| Ad lib. + A.P.E. | 5 | 88 | 258 | 0.72 | 905 | 27.3 | 4 | 86 | 264 | 0.71 | 836 | 20.7 |
| Control | 4 | 0 | 120 | 0.71 | 762 | 24.7 | | | | | | |
| Pair-fed + A.P.E. | 4 | 3 | 121 | 0.71 | 903 | 14.8 | | | | | | |

With only three injections of the A.P.E., the protein katabolism of the fasting rats was markedly decreased. After 10 days' treatment (male rats fed ad libitum) the fraction of the total heat derived from protein katabolism was practically the same for the treated and the control rats. In all other comparisons, when the treatment extended beyond 10 days the protein katabolism of fast was increased in the rats treated with the A.P.E.

The over-all statistics of table 4 give the cumulative results of all measurements in each group of rats for the three fasting periods and indicate the effect of the A.P.E. on the relationship of heat production to body weight. The data yielded by the rats injected for only 3 days with the A.P.E. were not included in these calculations, so the data reported represent the conditions prevailing during the time in which the treated rats were in a state of nitrogen plethora as evidenced by the excretion of excess urinary N during fast.

The correlation of hourly fasting heat production and body weight was considerably higher in the control rats than in those treated with the A.P.E. The regression coefficients expressing the average change in fasting heat production for unit change in body weight were 1.88 cal./hr./gm. in the male control rats, and 1.59 cal./hr./gm. in the female control rats. The digression from these values was most marked with the treated rat fed ad libitum, in which the regression of heat production on body weight was only 0.55 cal./hr./gm. for the males and 0.52 cal./hr./gm. for the females. In the treated rats pair-fed with the controls, the regression coefficient was decreased about 50% in the males, but only 10% in the females, the latter difference being non-significant.

Adjustment of the fasting heat production to a standard body weight of 200 gm., on the basis of the respective regression coefficients, indicated that the net effect of the A.P.E. on the total heat was negligible in the males, while in the females the total heat production was somewhat increased. However, the heat of protein katabolism was considerably increased in both sexes, and the values for this fraction of the

TABLE 4

Relation of fasting heat production to body weight of rats as affected by daily injections of A.P.E.

| Category of interest | Control Rats | Pair-fed Rats + A.P.E. | Ad libitum + A.P.E. |
|---|---|---|---|
| **Males** | | | |
| Number of measurements | 15 | 14 | 15 |
| Mean body weight (range), gm. | 184 (96–298) | 179 (102–278) | 185 (94–312) |
| Mean heat production (range), cal./hr. | 889 (691–1187) | 886 (801–1150) | 900 (790–1100) |
| Correlation coefficient | 0.93 | 0.56 | 0.45 |
| Regression of total heat on body weight[1] | 1.88 ± 0.20 | 0.97 ± 0.39 | 0.55 ± 0.30 |
| Regression of protein heat on body weight | 0.46 | 0.37 | 0.91 |
| Regression of non-protein heat on body weight | 1.42 | 0.60 | —0.36 |
| Total heat production at 200 gm. weight, cal./hr. | 919 | 906 | 908 |
| Protein heat production at 200 gm. weight, cal./hr. | 138 | 191 | 221 |
| Non-protein heat production at 200 gm. weight, cal./hr. | 781 | 715 | 687 |
| **Females** | | | |
| Number of measurements | 15 | 15 | 12 |
| Mean body weight (range), gm. | 150 (104–209) | 172 (108–247) | 201 (125–299) |
| Mean heat production (range), cal./hr. | 752 (644–860) | 887 (736–1058) | 886 (667–1045) |
| Correlation coefficient | 0.82 | 0.72 | 0.27 |
| Regression of total heat on body weight[1] | 1.59 ± 0.31 | 1.44 ± 0.39 | 0.52 ± 0.16 |
| Regression of protein heat on body weight | 0.31 | 0.07 | 0.14 |
| Regression of non-protein heat on body weight | 1.28 | 1.37 | 0.38 |
| Total heat production at 200 gm. weight, cal./hr. | 832 | 927 | 885 |
| Protein heat production at 200 gm. weight, cal./hr. | 137 | 193 | 224 |
| Non-protein heat production at 200 gm. weight, cal./hr. | 695 | 734 | 661 |

[1]Cal. per hr. per gm. body weight.

total heat were remarkably similar for both male and female rats in each particular group. The extra protein katabolism of the treated rats fed ad libitum was larger than that of the treated rats pair-fed with the controls.

In the treated male rats the non-protein fraction of the fasting heat was decreased to about the same extent that the protein fraction was increased, so the net difference in total heat was negligible.

### DISCUSSION

The saline A.P.E. used in the present investigation appeared to contain two principles affecting metabolism: one was active in promoting the increase in body substances (growth and conservation of protein); the other acted, directly or indirectly, as a stimulant of cellular metabolism.

After extended treatment an antagonistic agent developed, particularly in the male rats, which opposed both of these effects. The initial responses of the sexes were quite similar, but with progressive treatment the activity of the A.P.E. was counteracted in the male rats, and after 5 to 6 weeks the antagonistic agent became dominant over the A.P.E., as evidenced by the waning conservation of dietary protein, the decreased basal metabolic rate, the retardation of growth, and the depression of appetite.

The conservation of dietary protein persisted over an extended period of treatment with the A.P.E. The protein katabolism of fasting rats treated with the A.P.E. was decreased initially, but after 10 days the fasting protein katabolism was increased. These observations can be explained as the result of the accumulation of surplus protein. As the conservation of dietary protein persisted and the body stores increased, some of the extra supply was preferentially katabolized during fast, thus sparing the oxidation of fat as a source of energy for the increased metabolic rate. Consequently, after 2 weeks or more, the percentage of the total heat produced from protein by the rats treated with the

A.P.E. during fast was greater than that in the controls, and the protein sparing effect of the A.P.E. was obscured.

In view of the development of the antagonistic principles and the protein plethora, it appears that the limited treatment (3 days) with the A.P.E. afforded a more representative picture of the functioning of the anterior pituitary in the normal rat than did the extended treatment. The limited treatment under conditions of controlled feeding was sufficient to obviate the immediate adjustmental reactions and to reveal the essential regulatory nature of the principles in the saline A.P.E.

The increased heat production in the treated rats appeared to result from a true stimulation of cellular metabolism, since the extra heat was not correlated with body weight, and since the energy required for the accelerated metabolism was derived from the nutrient which happened to be most available at the time. Under normal dietary conditions carbohydrate provided the extra energy. During fast the extra energy was derived from fat, until the accumulation of surplus protein was such that protein katabolism increased and fat was spared.

The correlation and regression coefficients for the fasting heat production and body weight might be interpreted to mean that the weight changes (increase or decrease) induced by the A.P.E. involved the gain or loss of body substance which was less active, metabolically, than was the equivalent body substance in the control rats. This observation tends to confirm that of Lee and Gagnon ('30) and Kleiber and Cole ('39), with respect to the effect of the growth hormone on metabolism, but militates against the conclusion of the latter authors that the somatic adaptation of tissue metabolism is in correlation to body size.

As an alternate interpretation the low correlation and regression coefficients for the heat production and body weight in the rats treated with the A.P.E. may reflect a developing activity of antagonistic principles which counter-

acted the accelerating effect of the A.P.E. on metabolism in the earlier stages of treatment.

The results reported herein most closely agree with those obtained by Gaebler ('33) with dogs. In his subsequent investigations the calorigenic and protein sparing effects of the A.P.E. have been reproduced in thyroparathyroidectomized dogs (Gaebler, '35), and during phlorizin or pancreatic diabetes (Gaebler and Zimmerman, '39; Gaebler and Galbraith, '41). The results seem thus to be a direct effect of the A.P.E., rather than an indirect action mediated by the thyroid or pancreas.

In general, the results reported herein corroborate and extend the observations of Lee and Schaffer ('34), Schaffer and Lee ('35), Harrison and Long ('40) and Paschkis ('42) with respect to the effect of the A.P.E. on protein metabolism.

### SUMMARY

The effects of daily treatment of normal young male and female rats with saline anterior pituitary extract (A.P.E.) over a period of 12 weeks was investigated with respect to the energy metabolism of protein and non-protein nutrients, and to the progressive changes of fasting metabolism relative to body weight and sex.

The total heat production under conditions of normal nutrition was increased by the A.P.E. The net increase was the resultant of a diminished protein oxidation and a relatively larger increase in non-protein oxidation.

In male rats the fasting heat production was initially increased, but this effect gradually disappeared and was finally reversed. In female rats the fasting metabolism was increased throughout the period of observation. The heat of protein katabolism during fast was initially decreased, but after 2 weeks or more of treatment it was greater than that in the controls for both sexes. This was attributed to the development of a protein plethora.

The regression of the fasting heat on body weight indicated that the changes in body substance induced by the

A.P.E. (or its antagonist, in the male rats) were not as active, metabolically, as were tissue changes in untreated rats.

The A.P.E. appeared to act as a specific stimulant of cellular metabolism and, at the same time, was effective in promoting an increase in body substance which was less energetic than that assimilated normally. In the male rats an antagonistic agent developed which opposed both of these effects.

## LITERATURE CITED

FORBES, E. B., M. KRISS AND R. C. MILLER 1934 The energy metabolism of the albino rat in relation to the plane of nutrition. J. Nutrition, vol. 8, p. 535.

GAEBLER, O. H. 1933 Some effects of anterior pituitary extracts on nitrogen metabolism, water balance and energy metabolism. J. Exp. Med., vol. 57, p. 349.

———— 1935 Effects of thyroparathyroidectomy and carbohydrate intake on the action of anterior pituitary extracts. Am. J. Physiol., vol. 110, p. 584.

GAEBLER, O. H., AND H. W. GALBRAITH 1941 Effects of anterior pituitary preparations in experimental pancreatic diabetes. Endocrin., vol. 28, p. 171.

GAEBLER, O. H., AND W. J. ZIMMERMAN 1939 Effects of an anterior pituitary growth preparation on metabolism in phlorizin diabetes. Am. J. Physiol., vol. 128, p. 111.

HARRISON, H. C., AND C. N. H. LONG 1940 Effects of pituitary extracts in the fasted rat. Endocrin., vol. 26, p. 971.

KLEIBER, M., AND H. H. COLE 1939 Body size and energy metabolism in growth hormone rats. Am. J. Physiol., vol. 125, p. 747.

KRISS, M. 1938 The specific dynamic effects of proteins when added in different amounts to a maintenance ration. J. Nutrition, vol. 15, p. 565.

KRISS, M., AND L. VORIS 1937 A further contribution to the derivation of factors for computing the gaseous exchange and the heat production in the metabolism of proteins. J. Nutrition, vol. 14, p. 215.

LEE, M. O., AND J. GAGNON 1930 The effects of growth promoting and gonad stimulating principles of the anterior lobe of the pituitary on basal gaseous metabolism in the rat. Endocrin., vol. 14, p. 233.

LEE, M. O., AND N. K. SCHAFFER 1934 Anterior pituitary hormone and the composition of growth. J. Nutrition, vol. 7, p. 337.

PASCHKIS, K. E. 1942 Influence of anterior pituitary extract on protein and carbohydrate metabolism. Am. J. Physiol., vol. 136, p. 128.

SCHAFFER, N. K., AND M. LEE 1935 The effect of the anterior pituitary growth hormone on protein metabolism. J. Biol. Chem., vol. 108, p. 355.

VORIS, L., M. KRISS, L. F. MARCY AND R. S. BOWMAN 1942 Effects of prolonged daily treatment of normal rats with saline anterior pituitary extract. I. Sexual differences in appetite, growth and organ weights. J. Nutrition, vol. 24, p. 469.

# THE EFFECT OF VITAMIN B DEFICIENCY ON THE INTESTINAL ABSORPTION OF GALACTOSE IN THE RAT [1]

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The problem of intestinal absorption in vitamin B deficiency has attracted attention as being a possible explanation of the gastrointestinal dysfunction and anorexia that occur in such deficiency states (Pierce, Osgood and Polansky, '29; Gal, '30; Russell and Nasset, '41; Beams, Free and Glenn, '41; Harper, '42). The present study was undertaken in order to obtain further data on intestinal absorption in vitamin B complex deficiency.

## EXPERIMENTAL

Albino and piebald rats reared in the department colony were placed on a diet having a percentage composition of alcohol extracted casein, 18; butterfat, 8; sucrose or alcohol extracted starch, 68; Mendel-Hubbell-Wakeman salt mixture ('37), 4; and cod liver oil, 2. Approximately one-half of the animals received the sucrose diet and one-half received the starch diet. Each experimental animal was "paired" with a litter mate of the same sex which received the same diet with the addition of 5 gm. of brewer's yeast per 100 gm. of diet.

The food intake of the control member of the pair was regulated so that its weight was kept nearly the same as that of the experimental member. Pairs of animals of different ages were purposely used. After from 30 to 45 days on the diet the experimental animals showed both marked loss of weight and deficiency symptoms. At this time the studies of intestinal absorption were carried out.

The animals were fasted for 24 hours prior to the absorption study, water being withheld during the last 12 hours. A volume of 25% galactose solution sufficient to supply 6.0 gm. of galactose per kilogram of body weight was placed in a small dish in the animal's cage. In all cases the animals voluntarily drank all of the sugar solution in less than 3 minutes. Exactly 1 hour after the animal had started to drink the solution the jugular vein was severed and a sample of 0.1 cc. of blood was obtained which was diluted with 2.4 cc. of distilled water and subsequently analyzed for galactose. The animal was then decapitated, the abdomen opened, hemostats clamped on the lower esophagus and the pylorus, and the gastrointestinal tract removed. These operations consumed approximately 1 to 1½ minutes. The contents of the stomach and intestine were then washed quantitatively into separate containers with hot water and diluted to volumes of either 250 cc. or 500 cc. A portion of each solution was then saturated with dry picric acid, filtered, and the picric acid filtrate analyzed for sugar by the method of Myers and Bailey ('16). The colors were read in an Evelyn photoelectric colorimeter. Control studies in fasting rats gave negligible quantities of reducing substances by this method. Galactose was estimated by fermenting the diluted blood with baker's yeast and subsequently determining the non-fermentable reducing substance. In the present study the figures obtained for blood galactose include those for the non-fermentable reducing substance of blood as well as for galactose. However, numerous studies have indicated that the non-fermentable reducing substance of rat's blood is fairly constant and always amounts to less than 50 mg. per 100 cc.

## RESULTS

The results of absorption studies on thirty-eight pairs of animals in which the experimental member of each pair was deprived of the vitamin B complex are shown in table 1. This series contains both males and females and is made up of eighteen pairs of albino rats (indicated by W in the table) and twenty pairs of piebald rats (P). In both the deficient and control animals a considerable amount of the ingested sugar remained in the stomach. The average figure for the deficient rats is 35% of the ingested sugar, whereas the stomachs of the control animals contained 31% of the ingested galactose. The deficient rats had an average of 25% of the ingested sugar remaining in the intestine, whereas the control animals had an intestinal residue of 14% of the ingested galactose.

Absorption coefficients are expressed as milligrams of galactose absorbed per 100 gm. of body weight per hour. Some investigators maintain that the absorption coefficient can be more closely related to body surface than to body weight. In the present study this point is of no consequence since the deficient and control animals have approximately both the same body weight and body surface. The average absorption coefficient of the deficient animals is 244, whereas the average absorption coefficient of the control animals is 331. There is a considerable variation in absorption coefficient among the individual animals which is in part to be expected since the pairs are of different sexes, ages and weights and are of two different strains. In only six of the thirty-eight pairs does the absorption coefficient of the deficient animal exceed that of its litter-mate control.

Figures for blood galactose indicate an average value of 402 mg. per 100 cc. for the deficient animals, and 487 mg. per 100 cc. for the controls. In only three of the thirty-five pairs in which blood galactose estimations were obtained was the level of blood galactose of the deficient animal above that of its litter-mate control.

**TABLE 1**

*The effect of vitamin B complex deficiency on absorption coefficients and blood galactose levels.*

| Pair | Sex | Strain | Initial | Final | In stomach [1] | In intestines [1] | Absorption coefficient | In blood | Initial | Final | In stomach [1] | In intestines [1] | Absorption coefficient | In blood | Absorption coefficient | Blood galactose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | gm. | gm. | % | % | mg./100 gm./hr. | mg./100 cc. | gm. | gm. | % | % | mg./100 gm./hr. | mg./100 cc. | | |
| 1 | M | W | 64 | 33 | 12 | 48 | 236 | 330 | 50 | 37 | 4 | 35 | 368 | 360 | +132 | +30 |
| 3 | M | W | 79 | 58 | 29 | 27 | 266 | 502 | 69 | 56 | 38 | 11 | 308 | 548 | +42 | +46 |
| 4 | M | W | 115 | 69 | 48 | 12 | 238 | 408 | 127 | 69 | 9 | 38 | 315 | 484 | +77 | +76 |
| 6 | M | W | 85 | 50 | 9 | 45 | 278 | 412 | 62 | 48 | 33 | 6 | 367 | 484 | +89 | +72 |
| 7 | M | W | 112 | 77 | 48 | 8 | 263 | 354 | 104 | 60 | 9 | 31 | 362 | 570 | +99 | +216 |
| 10 | F | W | 103 | 58 | 42 | 26 | 195 | 346 | 97 | 71 | 51 | 10 | 238 | 408 | +43 | +62 |
| 11 | F | W | 117 | 70 | 24 | 15 | 366 | 476 | 112 | 70 | 24 | 11 | 390 | 570 | +24 | +94 |
| 12 | M | W | 181 | 106 | 60 | 16 | 146 | 126 | 177 | 113 | 40 | 18 | 253 | 188 | +107 | +62 |
| 14 | M | P | 77 | 50 | 27 | 36 | 220 | 480 | 78 | 56 | 21 | 11 | 409 | 730 | +189 | +250 |
| 15 | M | P | 61 | 48 | 24 | 12 | 384 | 556 | 40 | 45 | 23 | 7 | 418 | 556 | +34 | 0 |
| 16 | M | P | 60 | 45 | 21 | 17 | 374 | 552 | 41 | 46 | 40 | 10 | 304 | 488 | −70 | −64 |
| 17 | F | P | 51 | 43 | 16 | 10 | 447 | 708 | 45 | 42 | 26 | 9 | 394 | 580 | −53 | −128 |
| 18 | F | P | 70 | 44 | 31 | 48 | 129 | 328 | 72 | 55 | 46 | 8 | 275 | 440 | +146 | +112 |
| 19 | F | P | 74 | 52 | 49 | 21 | 184 | 368 | 59 | 55 | 14 | 13 | 435 | 700 | +251 | +332 |
| 20 | F | P | 45 | 29 | 26 | 36 | 224 | | 48 | 37 | 18 | 15 | 400 | | +176 | ... |
| 21 | F | P | 55 | 37 | 35 | 24 | 248 | 516 | 87 | 44 | 17 | 17 | 391 | 552 | +143 | +36 |
| 22 | F | P | 130 | 92 | 50 | 8 | 251 | 302 | 144 | 89 | 44 | 9 | 281 | 434 | +30 | +132 |
| 23 | F | P | 142 | 96 | 65 | 10 | 150 | 394 | 139 | 99 | 56 | 10 | 208 | 430 | +58 | +36 |
| 24 | F | P | 153 | 95 | 64 | 10 | 158 | 274 | 140 | 106 | 63 | 5 | 191 | 350 | +33 | +76 |
| 25 | F | P | 182 | 104 | 63 | 14 | 139 | 266 | 158 | 114 | 42 | 10 | 288 | 394 | +149 | +128 |
| 26 | F | P | 130 | 72 | 61 | 20 | 113 | 310 | 123 | 92 | 57 | 6 | 222 | 390 | +109 | +80 |
| 27 | M | W | 90 | 51 | 19 | 38 | 259 | 430 | 67 | 49 | 9 | 31 | 361 | 444 | +102 | +14 |
| 28 | M | W | 124 | 77 | 41 | 25 | 202 | | 109 | 75 | 16 | 10 | 443 | | +241 | ... |
| 29 | M | W | 73 | 49 | 12 | 35 | 317 | 452 | 78 | 43 | 35 | 12 | 316 | 390 | −1 | −62 |
| 30 | F | W | 83 | 47 | 18 | 36 | 275 | 400 | 93 | 53 | 27 | 10 | 380 | 560 | +105 | +160 |
| 31 | F | W | 67 | 43 | 14 | 40 | 277 | 448 | 60 | 49 | 17 | 10 | 437 | 502 | +160 | +54 |
| 32 | F | W | 111 | 70 | 40 | 30 | 182 | 346 | 112 | 83 | 41 | 9 | 303 | 488 | +121 | +142 |
| 33 | F | W | 73 | 46 | 10 | 56 | 204 | 452 | 65 | 47 | 20 | 12 | 406 | 570 | +202 | +118 |
| 34 | F | W | 88 | 59 | 16 | 43 | 248 | 416 | 88 | 57 | 24 | 14 | 368 | 502 | +120 | +86 |
| 35 | F | W | 72 | 52 | 52 | 9 | 237 | 390 | 72 | 43 | 11 | 16 | 440 | 458 | +203 | +68 |
| 36 | F | W | 59 | 39 | 23 | 24 | 321 | 372 | 74 | 42 | 6 | 31 | 374 | 580 | +53 | +208 |
| 38 | M | P | 43 | 35 | 20 | 30 | 300 | 502 | 57 | 45 | 6 | 9 | 510 | 766 | +210 | +264 |
| 41 | M | P | 192 | 119 | 42 | 22 | 221 | 318 | 203 | 118 | 53 | 17 | 175 | 324 | −46 | +6 |
| 42 | M | P | 124 | 87 | 59 | 10 | 188 | 302 | 100 | 90 | 54 | 6 | 241 | 400 | +53 | +98 |
| 43 | F | P | 37 | 30 | 9 | 38 | 317 | 484 | 32 | 30 | 29 | 15 | 334 | 498 | +17 | +14 |
| 45 | F | P | 107 | 74 | 41 | 13 | 274 | | 92 | 74 | 50 | 13 | 221 | | −53 | ... |
| 46 | F | P | 93 | 61 | 48 | 17 | 210 | 372 | 93 | 71 | 47 | 11 | 252 | 494 | +42 | +122 |
| 47 | F | P | 87 | 58 | 48 | 13 | 230 | 364 | 98 | 67 | 57 | 8 | 214 | 390 | −16 | +26 |
| Average | | | 95 | 61 | 35 | 25 | 244 | 402 | 91 | 64 | 31 | 14 | 331 | 487 | +87 | +85 |

[1] Fraction of ingested sugar found in the organ.

## DISCUSSION

The technic employed in the present study differs somewhat from that popularized by Cori ('25) although none of the variations are necessarily original. In some rats forced feeding by means of a stomach tube causes marked excitement accompanied by violent struggling. It is well-known that in humans such states of excitement are accompanied by alteration in gastrointestinal motility, and this is also undoubtedly true in rats. By having the rats spontaneously ingest the sugar any excitement is eliminated. Separate analyses of unabsorbed sugar in the stomach and intestine were made since it was felt that information regarding gastric evacuation of the sugar solution could be gained by this means and it was appreciated that this factor might greatly contribute to any results obtained. The amount and concentration of sugar fed have been reduced as much as possible. It is well-established that solutions of high osmotic pressure are not readily evacuated from the stomach, and in experiments which we have conducted on human subjects ingestion of strong sugar solutions frequently resulted in nausea and vague gastrointestinal distress. We feel that reduction in amount of sugar fed to such a level that the animals will voluntarily ingest the entire amount is desirable and outweighs advantages that would be afforded by a longer experimental period which would necessitate forced feeding of the rats.

Galactose was selected as a test sugar in these experiments since this sugar has been employed in this laboratory in indirect studies of intestinal absorption in dogs and humans (Beams, Free and Glenn, '41; Free, Leonards and Myers, '42). This sugar is one which is selectively absorbed from the intestine at a rate greater than can be accounted for by simple diffusion. The results of Cori ('25) indicate that glucose and galactose have quite similar rates of absorption.

Cowgill, Deuel, Plummer and Messer ('26) noted that gastric atony was present in dogs with advanced vitamin B deficiency. Marked decrease in the rate at which vitamin B

deficient rats evacuated barium sulfate from the stomach was observed by Gal ('30) and by Gershon-Cohen, Shay and Fels ('41). Heublein, Thompson and Scully ('41) have reported that vitamin B complex deficiency caused marked retardation in gastric motility and moderate delay of small intestinal motility, and in our laboratory we have observed very marked gastric retention in dogs suffering from acute symptoms as a result of vitamin B complex deficiency. However, in the present study the data on gastric and intestinal residues of unabsorbed sugar indicate that, on the average, gastric emptying in the deficient rats has occurred at approximately the same rate as in the pair-fed controls since the average gastric retention in the deficient animals is 35% of the ingested dose, and in the controls, 31%. The residue of unabsorbed sugar in the intestines of the two groups indicates that the deficient animals did not absorb the galactose as rapidly as the control animals even though it was present in the intestine and available for absorption.

The actual values of the absorption coefficients are considerably higher than those obtained by Cori ('25) for galactose in control animals. It would appear that two factors contribute to these higher levels. In the first place, the average weight of the animals at the time of the absorption studies is considerably less than the weight of the rats employed by Cori. Furthermore, the rats in the present study had decreased in weight on an average of 30 gm. during the depletion period so that the ratio of intestinal surface to body weight was increased. The second factor which probably contributed to the greater absorption rate in our animals was the fact that the rats voluntarily ingested the sugar and did not suffer any fright or excitement as a result of the gavage.

Comparison of blood galactose elevation with the absorption coefficients indicates that the level of blood galactose 1 hour after ingestion of the galactose solution is a measure of the rate of absorption during the period. Different animals will have somewhat different rates of metabolism and excretion of the sugar but in general high blood galactose figures

are associated with high absorption coefficients and low blood galactose figures are associated with low absorption coefficients. In the thirty-five pairs of animals where blood galactose and absorption coefficients were obtained, there were five pairs in which the absorption coefficient of the deficient animal exceeded that of its control litter mate. In three of these pairs the level of blood galactose of the deficient animal also exceeded that of its litter mate. In only two pairs (nos. 41 and 47) did the values of blood galactose not correspond with the absorption coefficients.

In the present study no attempt was made to determine which member of the vitamin B complex contributed to the impairment in intestinal absorption. The animals in the present study were somewhat analogous to humans who subsist on vitamin B deficient diets which, rather than being deficient in a single member of the vitamin B complex, are deficient in most of them. However, they differ from human vitamin deficiency states in that restriction of vitamin B complex was complete in the animals and they developed acute symptoms in a short time. At the present time further studies are being made to ascertain which member or members of the vitamin B complex may be specifically involved in intestinal absorption.

Results of the present study do not indicate whether vitamin B complex deficiency has a specific action on the physico-chemical processes of absorption, or whether the effect is secondary to changes in the circulation or motility of the intestine.

## SUMMARY

Studies of the intestinal absorption of galactose were carried out in thirty-eight litter-mate pairs of rats in which one member of the pair received a diet deficient in the vitamin B complex, whereas the other member received the same diet supplemented with brewer's yeast. Intestinal absorption was determined by analysis of the unabsorbed sugar in the gastrointestinal tract 1 hour after its oral ingestion. The rate of intestinal absorption of the pair-fed litter-mate controls aver-

aged 35% more than that of the deficient animals. Analysis of the gastric residue in the animals indicated that the results were not affected by the rate of gastric emptying since this was approximately the same in the two groups of animals. Blood galactose levels 1 hour after the ingestion of the sugar were used as indirect measures of intestinal absorption. The blood galactose levels also indicated an impairment of intestinal absorption in the vitamin B complex deficient animals.

## LITERATURE CITED

BEAMS, A. J., A. H. FREE AND P. M. GLENN 1941 The absorption of galactose from the gastrointestinal tract in deficiency diseases. Am. J. Dig. Dis., vol. 8, p. 415.

CORI, C. F. 1925 The fate of sugar in the animal body. I. The rate of absorption of hexoses and pentoses from the intestinal tract. J. Biol. Chem., vol. 66, p. 691.

COWGILL, G. R., H. J. DEUEL, N. H. PLUMMER AND F. C. MESSER 1926 Studies in the physiology of vitamins. IV. Vitamin B in relation to gastric motility. Am. J. Physiol., vol. 77, p. 389.

FREE, A. H., J. R. LEONARDS AND V. C. MYERS 1942 Studies on the absorption and metabolism of galactose and glycine in dogs with vitamin B deficiency. Federation Proceedings, vol. 1, p. 110.

GAL, G. 1930 Die Störung der Resorption bei Mangel an Vitamin B. Biochem. Zeitschrift., vol. 225, p. 286.

GERSHON-COHEN, J., H. SHAY AND S. S. FELS 1941 $B_1$ avitaminosis. Roentgenologic studies of gastrointestinal tract in rats on vitamin $B_1$ deficiency diets. Am. J. Roentgenol., vol. 46, p. 876.

HARPER, H. A. 1942 Carbohydrate metabolism in thiamin deficiency. J. Biol. Chem., vol. 142, p. 239.

HEUBLEIN, G. W., W. D. THOMPSON AND J. P. SCULLY 1941 The effect of vitamin B complex deficiency on gastric emptying and small intestinal motility. Am. J. Roentgenol., vol. 46, p. 866.

MENDEL, L. B., R. B. HUBBELL AND A. J. WAKEMAN 1937 The influence of some commonly used salt mixtures upon growth and bone development of the albino rat. J. Nutrition, vol. 14, p. 261.

MYERS, V. C., AND C. V. BAILEY 1916 The Lewis and Benedict method for the estimation of blood sugar with some observations obtained in disease. J. Biol. Chem., vol. 24, p. 147.

PIERCE, H. B., H. S. OSGOOD AND J. B. POLANSKY 1929 The absorption of glucose from the alimentary tract of rats deprived of the vitamin B complex. J. Nutrition, vol. 1, p. 247.

RUSSELL, R. A., AND E. S. NASSET 1941 The effects of various vitamin supplements and of whole yeast on the digestion and absorption of the carbohydrate of a complete diet. J. Nutrition, vol. 22, p. 287.

# MEAD JOHNSON AND COMPANY
## 'B-COMPLEX' AWARD

Nominations are solicited for the 1943 Award of $1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute at Cleveland on April 7, 1943.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1st to December 31st the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more persons. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1942 must be in the hands of the Secretary by January 10th, 1943. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

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SECRETARY, AMERICAN INSTITUTE OF NUTRITION

# NUTRITION OF THE GUINEA PIG [1]

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Although the guinea pig has long been used in studies on
vitamin C, little has been done on the other nutritional re-
quirements of this animal. These requirements have been
studied in our laboratory since 1938, when the guinea pig was
adopted for assay of the grass juice factor (Kohler, Elvehjem
and Hart, '38). In an endeavor to replace the mineralized
winter milk used as the basal diet in this assay, recourse
was made to simplified dry rations which could be reproduced
at any time and place, and which would give greater latitude
to the investigation (Kohler, Randle, Elvehjem and Hart
('39).

Three groups of authors (Goettsch and Pappenheimer,
'31; Madsen, McCay and Maynard, '33, '35; Hogan and
Ritchie, '34) had previously reported experiments with
guinea pigs fed synthetic rations of the casein-dextrin type
containing varying amounts of yeast and supplemented with
sources of vitamins A, C, D, and E. All these rations sup-
ported good growth in rats. However, the first two groups
of investigators obtained poor or no growth with guinea pigs,

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the animals usually dying in a relatively short period. Hogan and Ritchie also found poor growth and survival on the same type of ration with guinea pigs but obtained good growth for considerable periods when the amount of yeast was increased to 15% (as against 5% to 8% used by the first two groups of authors) plus 2% of tikitiki.

Kohler et al. ('39) reported a ration, P-5 (table 1), likewise containing dextrin and yeast, which alone would not support

TABLE 1

*Rations used.*

| CONSTITUENTS | P-5 | S 25 | S 27 | S-28 |
|---|---|---|---|---|
| Dextrin, grams | 59 | | | |
| Sucrose, grams | | 74 | 74 | 62 |
| Casein (reprecipitated 4 times), grams | 18 | 18 | 18 | 30 |
| Liver extract powder 1–20, grams | | ? | | |
| Yeast (Pabst brewer's), grams | 6 | | | |
| Salts IV (Hegsted et al., '41), grams | 4 | 4 | 4 | 4 |
| Lard, grams | 12 | | | |
| Corn oil, grams | | 2 | 4 | 4 |
| Cod liver oil, grams | 1 | | | |
| Thiamine, micrograms | | 200 | 250 | 250 |
| Pyridoxine, micrograms | | 200 | 250 | 250 |
| Riboflavin, micrograms | | | 350 | 350 |
| Pantothenic acid, micrograms | | | 1000 | 1000 |
| Nicotinic acid, milligrams | | | 5 | 5 |
| Choline, milligrams | | | 400 | 400 |
| Ascorbic acid | 10 mg. per day | | | |
| α-Tocopherol acetate | 1 mg. per week | Fed by pipette | | |
| Haliver oil | 4 drops per week | | | |

guinea pigs, but which gave good growth after the addition of 20% of dried grass. They noted in several instances, however, that animals receiving this ration plus grass suddenly sickened during rapid growth and died. It was found that these animals had stomach ulcers. The authors postulated that this ration was deficient in at least two factors, namely, the grass juice factor and a factor necessary for the maintenance of the stomach lining.

The results to be reported in this paper have been obtained over a period of 3 years through the use of over 750 guinea

pigs. During this time the approach has changed from the development of a ration for grass juice assay purposes to a study of the nutritional requirements of the guinea pig.

<div align="center">EXPERIMENTAL</div>

Guinea pigs were purchased from two local dealers during the first 2 years of this investigation. It finally became necessary to obtain guinea pigs from a large commercial breeder. All pigs were received at weights ranging from 200–300 gm. and were started immediately on experiment. They were distributed equitably as regards weight and sex among the groups comprising a given run. In most of the work five animals were employed per group.

The guinea pigs were kept in a humidity-controlled, air-conditioned room at 80° ± 2°F. The pigs were housed in metal cages, 15 inches by 20 inches (five pigs) or 10 inches by 15 inches (one pig), with $\frac{1}{2}$ inch mesh screen bottoms. The animals were weighed three times weekly and fed and watered daily.

Water and ration were fed in 1-pound butter jars during the first $2\frac{1}{2}$ years. Recently water bottles and metal hoppers for the ration have been substituted in an effort to reduce coprophagy. The composition of the rations is shown in table 1.

When the basal ration was supplemented with appreciable amounts of dry material, the supplements were mixed into the ration at the designated level and the sucrose content was correspondingly decreased. The yeast [2] and dried grass [3] were stored at −7°C. until used and were checked from time to time for grass juice factor activity by the mineralized winter milk assay (Kohler et al., '38; Randle et al., '40). The rations were mixed weekly and stored in the refrigerator.

Supplements amounting to less than 2 ml. per day were fed by pipette. Halibut liver oil, ascorbic acid, α-tocopherol

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[2] The yeast used to supplement the sucrose rations was in all cases Northwestern Yeast Foam.

[3] From the Cerophyl Laboratories.

acetate and concentrated milk fractions were fed in this manner. When the amount of milk or grass juice was too large to be fed by pipette, the supplement was introduced into the cage in a small dish, and the pigs deprived of water until the supplement was consumed. At the conclusion of the experimental period all the surviving animals were sacrificed and autopsied.

<div align="center">RESULTS</div>

Much of the preliminary work is not included and only the more significant studies are reported in table 2. These results are expressed for a 7-week test period, though some animals have been continued longer on the experiment. The great majority of the experiments have been repeated several times and the approximate number of trials may be derived from table 2 by dividing the number of animals by 5. Whenever possible, groups and rations which were essentially equivalent have been combined for conciseness. The total growth has varied from run to run, but the growth differences within a single run, due to a given supplement, were always clearly discernible.

<div align="center">

*Dextrin rations P-5*

</div>

The addition of 10% of grass (or equivalent amounts of grass juice) to ration P-5 (table 1) produced an appreciable growth response which, however, was not as great as that described by Kohler et al. ('39) who used this ration supplemented with 20% of dried grass. The feeding of 20 ml. of winter milk, low in the grass juice factor, with ration P-5 + 10% grass, not only tended to decrease the incidence of stomach ulcers, but also increased the rate of growth. This would suggest that P-5 was lacking not only in the factor supplied by grass but also in a factor supplied by milk. However, since the addition of winter milk alone to ration P-5 produced considerable growth, it was concluded that this ration must have contained some source of the grass juice factor. Ration P-5 was therefore discontinued and work was initiated with rations of a more synthetic nature.

TABLE 2

*Growth of guinea pigs on synthetic rations.*

| RATION PLUS SUPPLEMENTS | ANIMALS PER GROUP [1] | SURVIVORS AT 7-WEEKS | AVERAGE SURVIVAL [1] | AVERAGE GROWTH [2] |
|---|---|---|---|---|
| | | | | *gm /day* |
| P-5 | 19 | 8 | 81 | 0.1 |
| P-5 + 20 ml. milk | 45 | 35 | 88 | 1.8 |
| P-5 + 10% grass or ⌀ amount of grass juice | 18 | 12 | 85 | 2.0 |
| P-5 + 10% grass + 20 ml. milk | 12 | 7 | 86 | 3.2 |
| S-25 | 30 | 14 | 81 | —0.8 |
| S-25 + 20 ml. milk | 30 | 24 | 92 | —0.1 |
| S-25 + 10% grass | 15 | 11 | 86 | 1.2 |
| S-25 + fresh grass — 20 gm./day | 6 | 6 | 100 | 1.9 |
| S-25 + grass juice ⌀ 20 gm./day fresh grass | 23 | 9 | 68 | —0.2 |
| S-27 | 8 | 0 | 52 | |
| S-27 + 20 ml. milk | 9 | 4 | 80 | 0.4 |
| S-27 + 8% yeast | 8 | 3 | 74 | 0.6 |
| S-27 + 8% yeast + 20 ml. milk | 9 | 6 | 86 | 1.5 |
| S-27 + 8% grass | 8 | 3 | 67 | 0.7 |
| S-27 + 8% yeast + 8% grass | 9 | 3 | 72 | 1.5 |
| S-27 + 8% yeast + 8% grass + 20 ml. milk | 9 | 8 | 96 | 3.0 |
| S-27 + 4% yeast + 4% grass + 20 ml. milk | 10 | 7 | 97 | 1.6 |
| S-28 | 20 | 1 | 57 | |
| S-28 + 20 ml. milk | 14 | 9 | 85 | 0.8 |
| S-28 + 8% yeast | 6 | 4 | 84 | 1.1 |
| S-28 + 8% yeast + 20 ml. milk | 10 | 5 | 89 | 0.9 |
| S-28 + 16% yeast | 4 | 3 | 72 | 0.7 |
| S-28 + 16% yeast + 20 ml. milk | 5 | 5 | 100 | 2.3 |
| S-28 + 8% grass | 10 | 1 | 60 | 0.8 |
| S-28 + 8% grass + 20 ml. milk | 15 | 8 | 87 | 2.1 |
| S-28 + 16% grass | 10 | 7 | 83 | 2.0 |
| S-28 + 16% grass + 20 ml. milk | 15 | 9 | 84 | 2.1 |
| S-28 + 4% yeast + 4% grass | 63 | 24 | 75 | 1.0 |
| S-28 + 4% yeast + 4% grass + 20 ml. milk | 72 | 49 | 86 | 1.8 |
| S-28 + 8% yeast + 8% grass | 24 | 16 | 89 | 2.1 |
| S-28 + 8% yeast + 8% grass + 20 ml. milk | 19 | 18 | 98 | 3.1 |
| S-28 + 16% yeast + 16% grass | 4 | 3 | 97 | 2.2 |
| S-28 + 16% yeast + 16% grass + 20 ml. milk | 5 | 5 | 100 | 3.8 |
| S-28 + 10% whole dried kidney | 5 | 0 | 55 | |
| S-28 + 10% kidney + 8% grass | 9 | 3 | 83 | 0.7 |
| S-28 + 10% kidney + 8% grass + 20 ml. milk | 9 | 3 | 78 | 1.5 |

[1] Only those animals are included which survived the first 2 weeks. Average survival = $\frac{\text{weeks survived}}{7 \text{ weeks}} \times 100.$

[2] Calculated by subtracting the second week's average weight from the seventh week's average weight and dividing by 42. Only those animals which survived 7 weeks are included in the average growth column.

## Sucrose rations

These rations were patterned after the highly purified diets widely used in rat experiments. S-25 (table 1), a ration producing good growth when fed to rats (Black, Frost and Elvehjem, '40; Unna, Richards and Sampson, '41), would not support guinea pigs for any length of time. The addition of either milk or grass to S-25 increased the survival of the guinea pigs, but in the case of the milk addition there was no significant increase in growth. Grass induced some growth but the response obtained was moderate. Since only 2% of liver extract powder was included in S-25, the B complex content of the ration might have been the limiting factor. However, raising the liver extract to 4% increased the survival only slightly and did not appear to have much effect on growth. Other workers in this laboratory (Henderson, McIntire, Waisman and Elvehjem, '42) had found that they were able to raise albino rats to maturity and to obtain several generations on a synthetic ration in which the vitamin requirements were supplied by pure crystalline compounds and halibut liver oil. This ration, S-27 (table 1), was used in further investigations into the nutritional requirements of the guinea pig. Ration S-27 was unable to support growth in guinea pigs, all dying within 4 weeks. The addition to this basal ration of either 8% of dried grass, 8% of yeast or 20 ml. of winter milk resulted in slight growth responses and definitely prolonged survival. A combination of 8% of yeast and 8% of grass resulted in an additive growth response. However, when 20 ml. of winter milk were added to the basal ration S-27 + 8% of yeast + 8% of grass, even better growth resulted. The various combinations of the three supplements indicated that only in the presence of all three was the best growth obtained (table 2).

In order to preclude the stimulatory effect of milk being due to its protein or amino acid content, the amount of casein in the ration was raised from 18% to 30% (ration S-28, table 1). This ration did not permit guinea pigs to survive

for any longer period than did S-27, nor did the increased protein level remove the need for either yeast, grass, or milk. However, the hair coat and general condition of the animals appeared to be better and therefore S-28 was substituted as the basal ration in further studies. Since it has been reported that cystine may be a limiting factor for growth of the rat when casein is the sole protein source (Mulford and Griffith, '42), 0.1% of cystine was added to various combinations of the basal ration supplemented with 4% of yeast, 4% of grass, and 20 ml. of winter milk. The addition of cystine did not exert a beneficial effect. Half of the casein in S-28 was replaced by wheat gluten to test the effect of another source of protein with a different distribution of amino acids. No beneficial effect of wheat gluten was observed when this modified ration was fed with and without yeast, grass and winter milk in all the possible combinations.

Higher levels of individual supplements were fed. Sixteen per cent of either yeast or grass with and without milk were added to the basal ration. Even at these levels none of the individual supplements supplied all of the growth essentials for the guinea pig, since the addition of milk to these high levels of yeast or grass resulted in improvement of growth and survival. In the presence of both 16% of grass and 16% of yeast the addition of 20 ml. of winter milk caused an increase in growth from 2.2 gm. to 3.8 gm. per day for a period of 7 weeks (table 2).

The chick, a more rapidly growing animal than the rat, has been shown to require some factors supplied by whole dried kidney that are not required by the dog or the rat (Hegsted, Mills, Elvehjem and Hart, '41). Supplementing S-28 with 10% dried whole kidney failed to make it adequate for normal growth or development of guinea pigs. Even in the presence of 8% of grass and 20 ml. of winter milk, the addition of whole dried kidney was unable to produce maximum growth.

Since the growth response by the addition of 20 ml. of milk to either S-28 + 4% of yeast + 4% of grass, or S-28 + 8% of yeast + 8% of grass was quite appreciable and consistent, these two rations were used as assay rations for preliminary fractionation of winter milk. It was found that the factor contained in winter milk was water soluble, since it was present in the skim milk and whey fractions of milk and not in the butterfat fractions. From evidence at hand it appears that the factor was not precipitated by four volumes of 95% ethyl alcohol, nor was it extracted from whey by continuous ether extraction for 48 hours. It was stable to autoclaving for 1 hour.

In an attempt to identify the factor present in milk, the newer growth factors suggested for various species were added either singly or in combination to the basal ration, and to the basal ration plus 4% of yeast and 4% of grass in the presence and absence of the milk supplement. The following substances fed daily had no consistent effect: 40 mg. inositol, 80 mg. asparagine, 1 mg. glutamine, 600 µg. traumatic acid, and 600 µg. para-aminobenzoic acid.

## DISCUSSION

Relatively little is known of the performance of herbivora on diets of known chemical composition since the rat has always been the conventional experimental animal in nutritional research. Since this investigation was initiated, several workers have published studies on guinea pigs fed sugar-casein rations. Similar to the results obtained here, they found that rations so supplemented as to produce good growth in the rat were inadequate for growth and maintenance in the guinea pig. Cannon and Emerson ('39) and Clark ('41) utilized 10% of yeast or yeast extract to supply the B complex. Both reported, however, the necessity of adding grass, but neither mentioned the need of factors other than those found in grass. Cannon and Emerson used a short experimental period and were concerned only with the immediate responses to crude foods or fractions fed. Clark used much larger animals, and his ration (no. 4) also differed in that it

contained soybean oil. Woolley ('42) has reported evidence indicating that guinea pigs require at least three dietary essentials besides those now available in pure form, namely, dried grass, a 50% ethyl alcohol soluble and a 50% ethyl alcohol insoluble fraction of linseed oil meal.

The growth reported by Clark ('41) on ration 5 and by Kohler et al. ('39) may be explained by the high level of dried grass included in their rations, since guinea pigs can grow well and reproduce when whole dried grass is their only food (Kohler, private communication). However, probably equally important is the presence of dextrin in their rations. It has long been known that rats feeding on dextrin can obtain more of the B-complex through coprophagy than those feeding on sucrose (Guerrant, Dutcher and Tomey, '35). Cannon and Emerson ('39) reported preliminary experiments indicating that guinea pigs on dextrin-containing rations would grow for considerable periods without grass. Since coprophagy is frequent in guinea pigs, it seems quite possible that, in the presence of dextrin, this species may obtain much of their special requirements from an enteric source.

The evidence presented here indicates that our guinea pigs fed a sucrose, casein, salt mixture, corn oil ration, supplemented with halibut liver oil and the known crystalline vitamins, require additional factors present in yeast, grass, and milk. The interrelationship of the three factors is illustrated in table 2. It can be seen that the single addition of milk, grass, or yeast to the basal ration, although increasing considerably the survival time, had little effect on growth. Combination of any two of these substances resulted in somewhat improved growth, but only after all three were simultaneously added to the ration, was an approximation to normal growth obtained.

With the exception of vitamins A, C, and E, very little is known of the quantitative requirements of the guinea pig for the crystalline vitamins. These were fed at a level above that known to be sufficient for the rat. Materials which are rich sources of the B complex, e.g., liver and kidney, were

added in amounts that would double, if not triple, the total vitamin content of the ration. The addition of these substances to the basal ration did not nullify the effect of the subsequent addition of yeast, milk, or grass.

The nutrient factors contributed by yeast, milk or grass are probably not identical with the supplementary factors required by the rat and may not be identical with the additional factors required by the chick. Four per cent of liver extract powder added to similar synthetic rations will produce maximal growth in rats (Black et al., '40; Unna et al., '41). A supplement of 10% of dried kidney to the same ration will supply all of the chick requirements (Hegsted et al., '40). Neither of these materials could replace yeast, grass, or milk, as supplements promoting the growth of guinea pigs on S-28.

Kohler et al. ('39), Cannon and Emerson ('39) and Clark ('41) all postulated that the factor in grass required by guinea pigs on dry rations was the same as that described by Kohler et al. ('36, '37, '38) and Randle et al. ('40), namely, the grass juice factor. It is our belief that this is true. However, in addition to the grass juice factor, "folic acid"[4] abundant in fresh grass and grass juice may also be involved, as may biotin.

Some precedent for a special factor in yeast exists in the literature. Hogan and Ritchie ('34), Kohler and co-workers ('39), Cannon and Emerson ('39) and Clark ('41) included yeast or yeast extract in their rations. As yet, neither biotin nor "folic acid" has been excluded as the factor or factors present in yeast. Now that crystalline biotin has been made available its possible application to guinea pig nutrition may be investigated.

The results reported in this paper indicate that the factor supplied by milk is not a protein constituent. Yeast has been fed to guinea pigs at a level of 16% and since yeast is approximately 40% protein, the total protein content of the ration was approximately 37%. It seems highly improbable that the guinea pig would require amounts of amino acid that were not supplied by a mixture of 30% casein and 16% dried yeast.

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[4] The "folic acid" assays were kindly performed by Dr. Brian L. Hutchings.

Although rigorous proof of the non-identity of biotin or "folic acid" with the factor contained in 20 ml. of winter milk has as yet not been obtained in this laboratory, presumptive evidence exists. Winter milk has been assayed by the methods of Hutchings, Bohonos and Peterson ('41) and found to be very low in folic acid activity. The casein that is used in the ration (reprecipitated four times) is relatively unpurified. It might be expected that if the milk contained large amounts of biotin, the casein would carry sufficient biotin for the needs of the guinea pig.

### SUMMARY

Evidence is presented for the existence of three factors required by the guinea pig in addition to the factors that have been demonstrated to be required by the rat and the chick; namely:

1. A factor or factors supplied by 16% of grass which is not supplied in adequate amounts by 16% of yeast and 20 ml. of winter milk.

2. Some factor or factors supplied by 16% of yeast which is not supplied in adequate amounts by 16% of grass and 20 ml. of winter milk.

3. Some factor or factors supplied by 20 ml. of winter milk which is not supplied in adequate amounts by 16% of grass and 16% of yeast.

After the preparation of this paper an article by Hogan and Hamilton appeared (Hogan, A. G., and Hamilton, J. W., J. Nutrition, vol. 23, p. 533, 1942). These authors successfully reared guinea pigs on simplified rations which contained dried yeast as the source of all the water-soluble vitamins. They are of the impression that, because of their success with yeast, the non-existence of what we have called the "grass juice factor" is probable. It should be pointed out that we have unpublished data on the distribution of the "grass juice factor" showing that it is contained in yeasts as measured by the winter milk assay, and that the particular yeast we used [5] in this study is a fair source of this factor.

[5] See footnote 2, page 505.

## LITERATURE CITED

BLACK, S., D. V. FROST AND C. A. ELVEHJEM 1940 The relationship of vitamin B₆ and pantothenic acid to factor W studies. J. Biol. Chem., vol. 132, p. 65.

CANNON, M. D., AND G. A. EMERSON 1939 Dietary requirements of the guinea pig with reference to the need for a special factor. J. Nutrition, vol. 18, p. 155.

CLARK, W. G. 1941 Maintenance of adrenalectomized guinea pigs. Proc. Soc. Exp. Biol. and Med., vol. 46, p. 253.

GOETTSCH, M., AND A. M. PAPPENHEIMER 1931 Nutritional muscular dystrophy in the guinea pig and rabbit. J. Exp. Med., vol. 54, p. 145.

GUERRANT, N. B., R. A. DUTCHER AND L. F. TOMEY 1935 The effect of the type of carbohydrate on the synthesis of the B vitamins in the digestive tract of the rat. J. Biol. Chem., vol. 110, p. 233.

HEGSTED, D. M., J. J. OLESON, C. A. ELVEHJEM AND E. B. HART 1940 The essential nature of a new growth factor and vitamin B₆ for chicks. Poultry Science, vol. 19, p. 167.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., vol. 138, p. 459.

HENDERSON, L. M., J. M. McINTIRE, H. A. WAISMAN AND C. A. ELVEHJEM 1942 Pantothenic acid in the nutrition of the rat. J. Nutrition, vol. 23, p. 47.

HOGAN, A. G., AND W. S. RITCHIE 1934 Nutritional requirements of rabbits and guinea pigs. Missouri Agr. Exp. Sta. Res. Bul. 219.

HUTCHINGS, B. L., N. BOHONOS AND W. H. PETERSON 1941 Purification and properties of an eluate factor required by certain lactic acid bacteria. J. Biol. Chem., vol. 141, p. 521.

KOHLER, G. O., C. A. ELVEHJEM AND E. B. HART 1936 Growth stimulating properties of grass juice. Science, vol. 83, p. 445.

————— 1937 Further studies on the growth promoting factors associated with summer milk. J. Nutrition, vol. 14, p. 131.

————— 1938 The relation of the "grass juice factor" to guinea pig nutrition. J. Nutrition, vol. 15, p. 445.

KOHLER, G. O., S. B. RANDLE, C. A. ELVEHJEM AND E. B. HART 1939 Simplified rations for guinea pigs suitable for assay of the grass juice factor. Proc. Soc. Exp. Biol. and Med., vol. 40, p. 154.

MADSEN, L. L., C. M. McCAY AND L. A. MAYNARD 1933 Possible relationship between cod liver oil and the muscular degeneration of herbivora fed synthetic diets. Proc. Soc. Exp. Biol. and Med., vol. 30, p. 1434.

————— 1935 Synthetic diets for herbivora with reference to the toxicity of cod liver oil. N. Y. Agr. Sta. Memoir No. 178.

MULFORD, D. J., AND W. H. GRIFFITH 1942 Choline metabolism. VIII. The relationship of cystine and methionine to the requirement of choline in young rats. J. Nutrition, vol. 23, p. 91.

RANDLE, S. B., H. A. SOBER AND G. O. KOHLER 1940 The distribution of the "grass juice factor" in plant and animal materials. J. Nutrition, vol. 20, p. 459.

UNNA, K., G. V. RICHARDS AND W. L. SAMPSON 1941 Studies on nutritional achromotrichia of rats. J. Nutrition, vol. 22, p. 553.

WOOLLEY, D. W. 1942 Some new dietary essentials required by guinea pigs. Federation proc., Am. Soc. of Exp. Biol., vol. 1, no. 1, part II, p. 193.

# PYRIDOXINE DEFICIENCY IN CHICKS

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Pyridoxine has been shown to be essential for the growth of chicks by several investigators (Carter and O'Brien, '39; Jukes, '39; Hegsted et al., '39, '40; Hogan et al., '41). Jukes ('39) also reported that pyridoxine-deficient chicks showed nervous symptoms, consisting of various convulsive movements. There have been no reports concerning the occurrence of fits and convulsions in chicks such as have been described for rats (Chick, El Sadr and Worden, '40; Lepkovsky, Krause and Dimick, '42; Daniel, Kline and Tolle, '42), dogs (Fouts, Helmer, Lepkovsky and Jukes, '38) and pigs (Chick, Macrae, Martin and Martin, '38). This may have been due to the use of experimental diets deficient in factors other than pyridoxine. We have, therefore, attempted to develop a pyridoxine-deficient diet for chicks which, in so far as is possible, is deficient only in pyridoxine so that uncomplicated pyridoxine deficiency may be studied.

The difficulty of preparing a pyridoxine-deficient diet for chicks involved the inclusion in the basal diet of all necessary factors other than pyridoxine. To do this, wheat bran and yeast, treated to remove pyridoxine, were included in the basal diet. The removal of the pyridoxine also removed other factors; among them were thiamine, riboflavin and choline. These three vitamins were added to the basal ration in synthetic form. By this treatment it was hoped to include in the basal diet a maximum number of essential factors for the chick other than pyridoxine.

## EXPERIMENTAL

*Care of the chicks.* Day-old White Leghorn chicks were fed a standard chick mash for 8 days before they were separated with respect to weight and started on the experimental diets. They were housed in electrically heated, wire-floored batteries and were given feed and water ad libitum.

*Treatment of yeast [1] and wheat bran.* The wheat bran was allowed to autolyze in water. Toluol was used as a preservative. The autolysis was carried out for a month at room temperature, after which the bran was extracted three times with water. A wine press was used to press out the water from the bran. Bakers' yeast was similarly autolyzed with water and toluol at room temperature for 3 weeks. The yeast was then extracted twice with methyl alcohol and the methyl alcohol removed from the extract by distillation at reduced pressure. The extracts of the wheat bran and yeast were separately acidified to pH 4.0 with concentrated sulfuric acid and treated five times with fuller's earth, the amount of fuller's earth used for each treatment being equivalent to half of the amount of estimated solids present in the extract. The fuller's earth was removed by filtration and the treated extracts were then concentrated at reduced pressure. The extracted yeast and wheat bran were dried and added to the diets with their fuller's earth-treated extracts in amounts equivalent to the original dry material.

*Preparation of the diet.* The basal diet contained the following per 100 gm.: extracted wheat bran + fuller's earth-treated wheat bran extract, 10; extracted yeast + fuller's earth-treated yeast extract, 5; water-washed casein, 15; gelatin, 5; glucose,[2] 50.45; calcium gluconate, 5; fish oil, 0.25; soybean oil, 3; cottonseed oil [3] containing 1% vitamin E distillate,[4] 2; sodium chloride mixture containing 0.49% manganese, 0.1% iron, 0.05% copper, 0.05% zinc, 0.05% aluminum,

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[1] Fresh bakers' yeast was donated for this work by Standard Brands, Inc.

[2] Cerelose.

[3] Wesson.

[4] The vitamin E distillate was donated by Distillation Products, Inc.

0.002% cobalt, and 0.04% iodine, 1; tricalcium phosphate, 2; dipotassium phosphate, 0.5; potassium chloride, 0.3; magnesium sulfate, 0.1; sodium silicate, 0.2; cholic acid, 0.1; choline, 0.1; hexane extract of alfalfa equivalent to 2% alfalfa; thiamine,[5] 0.5 mg.; and riboflavin,[5] 0.5 mg.

Ten groups of pyridoxine-deficient chicks totalling eighty-one were used, and another ten groups totalling fifty-eight chicks were fed the deficient diet plus 2 mg. per kilo of pyridoxine. Though there were some variations in the pyridoxine-deficient diets used, the behavior of the chicks was sufficiently uniform so that only one of the groups need be described in detail.

## RESULTS

After a small initial gain in weight (table 1) the pyridoxine-deficient chicks ceased to grow or grew very slowly. Out of the seven chicks in the illustrative groups under discussion,

TABLE 1

*The effect of pyridoxine upon growth and occurrence of convulsions in chicks. Illustrative data*

| DIET | NO CHICKS | AV WEIGHT AT START | AV WEIGHT AFTER 3 WKS ON DIET | NUMBER SURVIVORS | NO CHICKS OBSERVED IN CONVULSIONS |
|---|---|---|---|---|---|
| Basal | 7 | 64.4 | 83.8 | 5 | 7 |
| Basal + 2 mg. pyridoxine per kilogram | 8 | 65 0 | 179.7 | 8 | 0 |

two had convulsions after they were on the deficient diet for 12 days. On the fourteenth day one chick died in a convulsion. On the sixteenth day another chick died after several convulsions, the first of which occurred on the twelfth day. Convulsions in the remaining five chicks were occasionally observed until they had been on the diet for 24 days after which no convulsions were noted. The chicks were weak and, for the most part, squatted in the cages with wings slightly extended from their bodies, and their heads resting on the wire screen. The wing feathers seemed to grow faster than

the bodies of the chicks and sometimes extended beyond the tail feathers. There was little feather growth on the body, the down remaining for the most part. No signs of dermatitis were observed. Some of the positions taken by the chicks are shown in figure 1. At times some chicks shivered as though
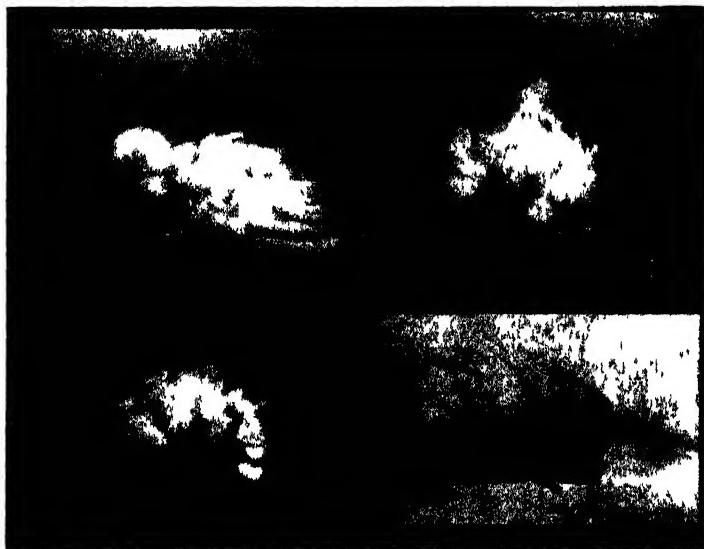


Fig. 1   Positions assumed by pyridoxine deficient chicks.

they were cold, but the chicks themselves, as well as their surroundings were warm. The tails of some chicks vibrated very rapidly. The chicks walked with a stiff high-stepping gait.

After 32 days, one of the remaining five chicks died, and two others died after 45 days. At this time the remaining two chicks were given 1 mg. each of pyridoxine. One died and the other gained 26 gm. in 2 days, after which it went into a continuous convulsion which lasted until the chick died about 2 days later.

The control chicks fed 2 mg. pyridoxine [6] per kilo of diet grew rapidly (table 1) and appeared normal for about 17

[6] The pyridoxine was donated by Merck and Co.

days, after which the rate of growth decreased in some of them. They were discarded after they were on the diet for 21 days. The control chicks were not quite normal, indicating the existence of a deficiency of a factor or factors other than pyridoxine.

*Pyridoxine-deficient syndrome.* The behavior of the pyridoxine-deficient chicks could well be divided into three different stages. In stage 1, all that could be noticed in the chicks was an abnormal excitability. Stage 2 was characterized by jerky, convulsive movements. Chicks would suddenly run aimlessly, often flapping their wings and keeping their heads down. Since the chicks were weak, these sudden, quick dashes were all the more surprising. In these dashes the chicks did not appear to be conscious of direction, since they often dashed off the end of a table. The eyes were apparently normal.

In stage 3 convulsions occur. While all the convulsions are not similar, they are nevertheless characteristic. Sometimes while resting on their breasts the chicks will raise their feet off the floor and flap their wings. Sometimes they will rapidly tap the floor with their feet while squatting. At times they will hop, largely with the assistance of their wings. During a convulsion they may fall on their sides or roll over on their backs and rapidly paddle with their feet. Their heads often jerk up and retract as in polyneuritis, sometimes move convulsively in an up-and-down movement, with the neck distended or twisted. The mandibles sometimes open and close rapidly. In severe convulsions, the chick enters a state of complete exhaustion which at times resembles a comatose state. After a period of time, varying from a few seconds to about 2 minutes, the chick usually recovers and becomes apparently normal. Convulsions will occur when the chicks are in the cages without any obvious disturbance. After their peculiar dashes, chicks will sometimes go into severe convulsions. In general, there seems no way of forecasting a convulsion, nor is there any certain method of bringing it about.

## DISCUSSION

The production of convulsions in pyridoxine-deficient chicks would seem to endow pyridoxine with a uniformity of function in different animals, similar to that of thiamine. It seems that before distinct convulsions occur, the basal ration must be relatively free of deficiencies other than that of pyridoxine. The complete absence of dermatitis is significant and is also in harmony with the decreased emphasis on dermatitis as a characteristic feature of pyridoxine-deficiency in rats (Lepkovsky and Krause, '42). The weakness of the deficient chicks and the shivering of some of them would seem to resemble the clinical findings of weakness (Spies, Bean and Ashe, '39) and paralysis agitans (Jolliffe, '41) in the human.

The promptness with which convulsions occur in chicks, after about 12 days on the deficient diet, is in striking contrast to the 4 to 6 months that is required by rats to develop similar convulsions.

## SUMMARY

1. The preparation for chicks of a diet deficient chiefly in pyridoxine has been described.

2. Pyridoxine deficiency in chicks is characterized by slow growth, convulsions, and other nervous manifestations.

3. No dermatitis of any kind was observed in the pyridoxine-deficient chicks.

## LITERATURE CITED

CARTER, C. W., AND J. R. O'BRIEN 1939 Vitamin B complex in relation to the nutrition of the chick and pigeon. Proc. Seventh World's Poultry Congress and Exposition, Cleveland, Ohio, p. 126.

CHICK, H., T. F. MACRAE, A. J. P. MARTIN AND C. J. MARTIN 1938 The water soluble B vitamins other than aneurin (vitamin $B_1$), riboflavin and nicotinic acid required by the pig. Biochem. J., vol. 32, p. 2207.

CHICK, H, M. M. EL SADR AND A. N. WORDEN 1940 Occurrence of fits of an epileptiform nature in rats maintained for long periods on a diet deprived of vitamin $B_6$. Biochem. J., vol. 34, p. 595.

DANIEL, E. P., O. L. KLINE AND C. D. TOLLE 1942 A convulsive syndrome in young rats associated with pyridoxine deficiency. J. Nutrition, vol. 23, p. 205.

FOUTS, P. J., O. M. HELMER, S. LEPKOVSKY AND T. H. JUKES 1938 Production of microcytic hypochromic anemia in puppies on synthetic diet deficient in rat antidermatitis factor (vitamin B$_6$). J. Nutrition, vol. 16, p. 197.

HEGSTED, D. M., J. J. OLESON, C. A. ELVEHJEM AND E. B. HART 1939 The "cartilage growth factor" and vitamin B$_6$ in the nutrition of chicks. J. Biol. Chem., vol. 130, p. 423.

———— 1940 The essential nature of a new growth factor and vitamin B$_6$ for chicks. Poul. Sci., vol. 19, p. 167.

HOGAN, A. G., L. R. RICHARDSON, H. PATRICK, B. L. O'DELL AND H. L. KEMPSTER 1941 Vitamin B$_6$ and chick nutrition. Poul. Sci., vol. 20, p. 180.

JOLLIFFE, N. 1941 Newer knowledge of the vitamin B complex. Bull. New York Acad. Med., vol. 17, p. 195.

JUKES, T. H. 1939 Vitamin B$_6$ deficiency in chicks. Proc. Soc. Exp. Biol. and Med., vol. 42, p. 180.

LEPKOVSKY, S., M. E. KRAUSE AND M. K. DIMICK 1942 The occurrence of fits in pyridoxine deficient rats. Science, vol. 95, p. 331.

LEPKOVSKY, S., AND M. E. KRAUSE 1942 Dermatitis in pyridoxine-deficient rats. Proc. Soc. Exp. Biol. and Med., vol. 49, p. 57.

SPIES, T. D., W. B. BEAN AND W. F. ASHE 1939 A note on the use of vitamin B$_6$ in human nutrition. J. Am. Med. Assoc., vol. 112, p. 2414.

# RESPONSE OF BACTERIA, YEAST AND RATS TO PEROXIDE-TREATED BIOTIN. INTESTINAL SYNTHESIS OF BIOTIN IN THE RAT [1]

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ONE FIGURE

Biotin has recently acquired an important position in animal nutrition because of its connection with so-called "egg white injury." Egg white injury was first encountered by Boas ('27) and studied extensively by Parsons ('31), Parsons and Kelly ('33), György ('39) and Birch and György ('39). These researches culminated in the brilliant discovery by du Vigneaud, Melville, György and Rose ('40) that biotin is the agent which counteracts the injurious effects of raw egg white. The egg white syndrome in rats resembles "pink disease" in man (Findlay and Stern, '29). Recently Sydenstricker, Singal, Briggs, De Vaughn and Isbell ('42) have reported an experimental biotin deficiency in humans, characterized by a scaly dermatitis, muscle pains and a change in the blood picture. Another aspect of nutrition involving biotin is the "spectacle eye" syndrome of the rat reported by Nielsen and Elvehjem ('41), a condition which has been studied extensively by several workers in relation to other vitamins (Oleson, Elvehjem and Hart, '40; Pavcek and Baum, '41). Biotin plays a role in fat synthesis and metabolism (Gavin and McHenry,

'41), and it has been noted that the biotin content of tumor tissue is higher than that of normal tissue (West and Woglam, '41). Studies of a dermatitis in chicks distinct from that produced by pantothenic acid deficiency have shown biotin to be involved in chick nutrition (Hegsted, Mills, Briggs, Elvehjem and Hart, '42). It is also important in the nutrition of the turkey (Patrick, Boucher, Dutcher and Knandel, '41).

In the nutrition of microorganisms biotin has held a first-rank place that antedates by several years recognition of its importance in the animal field. It was first discovered as a growth factor for yeast by Kögl and Tönnis ('36), but since that time has been found to be required by many bacteria and fungi (Robbins and Kavanagh, '41).

In developing an assay method for biotin by use of Lactobacillus casei, it was found that the constituents of the medium which had been rendered biotin-free by treatment with hydrogen peroxide showed marked biotin potency when tested by yeast. Because of this difference between the bacterial and yeast assays it seemed desirable to test the peroxide-treated biotin by animal assay. It has been found that the results with rats and with Lactobacillus casei agree in that neither organism shows any response to the peroxide-treated compound. Apparently yeast is able to regenerate biotin from the oxidation product. This is not surprising in view of the marked synthetic and reducing powers possessed by yeast. Biotin determinations on the feces and urine of the depleted rats showed such large amounts of biotin to be excreted that the work was extended to a study of the synthesis of biotin in the intestinal tract under various dietary conditions.

### EXPERIMENTAL

A biotin concentrate (no. 5000)[2] was used as a source of biotin for the comparative studies. A method of peroxide-treatment described by Shull, Hutchings and Peterson ('42) was used to produce an oxidation product of biotin. The biotin treated in this manner will be referred to hereafter as

[2] Supplied by S. M. A. Corp.

"peroxide-treated biotin." The peroxide treatment used in these experiments is milder than that employed by Brown and du Vigneaud ('41). In order to ascertain the effectiveness of this treatment, an aliquot was assayed both by the yeast method [3] of Snell, Eakin and Williams ('40) and by the Lactobacillus casei method. The data are given in table 1. From these results it appears that the use of oxidizing agents must be avoided in preparing biological materials for assay by the yeast method. Whether yeast responds to other decomposition products of biotin is a question that needs further study.

TABLE 1

*Response of Lactobacillus casei and yeast to peroxide-treated biotin.*
*(Biotin in mμg./cc.)*

| FORM OF BIOTIN | LACTOBACILLUS CASEI | | | YEAST | | |
|---|---|---|---|---|---|---|
| | Untreated | Treated | % Activity | Untreated | Treated | % Activity |
| Free acid | 0.3 | 0.0 | 0 | 0.3 | 0.3 | 100 |
| Free acid | 4.0 | 0.0 | 0 | 4.0 | 3.46 | 89 |
| Methyl ester | 125 | 0.0 | 0 | 125 | 102 | 82 |
| Concentrate (S.M.A.) | 3540 | 2.3 | < 1.0 | 4000 | 2220 | 55 |

The basal ration and the care of the animals have been described in a previous paper (Nielsen and Elvehjem, '41). In a preliminary experiment rats were placed on the basal ration modified by replacing 10% of casein with an equivalent amount of commercial egg white. After 5 weeks the egg white in the ration was reduced to 5% and kept at this level until the end of the experiment. The growth response resulting from the administration of biotin concentrates was considered to be the most accurate means to measure biotin activity. The growth curve for a typical animal appears in figure 1 as curve 1.

The growth noted over the feeding period is as follows: at the end of the fifth week the weight curve of the animal had begun to exhibit a plateau; then 1 μg. of "peroxide-treated

biotin'' was administered intraperitoneally, daily, for a period of 14 days, the rat losing 5 gm. during this time. This level of ''peroxide-treated biotin'' was then fed orally for 7 days and the animal's weight did not change. During the next 7 days the rat did not receive any form of biotin. Then 1 µg. of untreated biotin was fed orally for 7 days and over the week the rat showed a gain of 48 gm.
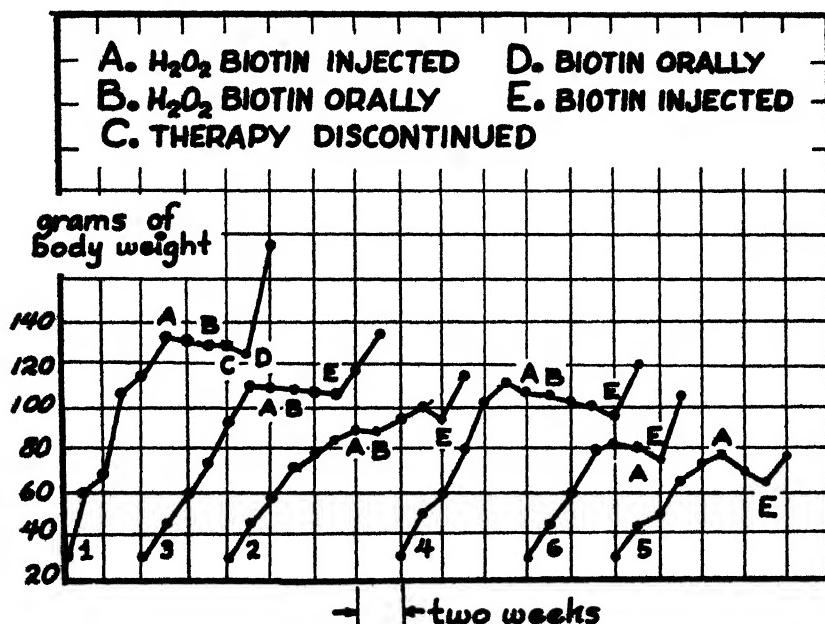


Fig. 1 Response of rats to various forms of biotin.

The ration for the five animals in the next experiment was the same as that described above, except that the commercial egg white was kept at a 10% level throughout the assay period. This level of egg white was adopted because a 5% level failed to tie up all the biotin synthesized in the intestinal tract. The first three rats were depleted of their stores of biotin over a 6-week period, then 1 µg. of ''peroxide-treated biotin'' was injected intraperitoneally for 7 days. Following this period ''peroxide-treated biotin'' was fed orally at the same level

for either 2 or 3 weeks, with the net result that all the animals were still losing or only maintaining weight. Then 1 μg. of biotin was injected daily for 7 days and marked gains in weight were obtained, as shown in figure 1, curves 2, 3 and 4.

The two additional rats were depleted for a 5-week period and then "peroxide-treated biotin" at a 1 μg. level per day was injected for 1 and 2 weeks, respectively. These two rats exhibited loss in weight, as previously observed. Then 1 μg. of biotin per day was injected and the usual responses were noted, as shown in curves 6 and 5.

From a study of the growth curves it appears that the rat is similar to Lactobacillus casei in that it cannot use this oxidized form of biotin either orally or parenterally.

The effect of oxidizing biotin, observed in this paper, is probably related to some compound less oxidized than the sulfone derivative of Hofmann, Melville and du Vigneaud ('41), since their compound does not promote the growth of yeast.

In order to study the synthesis of biotin in the intestinal tract of the rat a biotin-low ration was purified by the following procedure.

### Biotin-low ration

*Treatment of the components.* One thousand grams of acid-washed casein were suspended in 10 gallons of water and the pH adjusted to 8.5 with NaOH. To this viscous solution 430 ml. of Superoxol were added and the solution thoroughly stirred and allowed to stand at room temperature for 24 hours; then 230 gm. of powdered $MnO_2$ were added slowly and the solution stirred vigorously until no more oxygen was evolved. The solution was filtered by means of a filter press and the casein precipitated with acid. The casein was freed of excess acid by washing with warm water, collected on a cloth filter bed and finally dried and powdered in the usual manner.

The sugar was purified by extracting with 100% ethyl alcohol. One kilogram of sugar was placed in a cloth bag and

inserted in a Soxhlet extractor. Sufficient 100% ethyl alcohol was added to allow a continuous extraction for 30 hours.

The percentage composition of the ration and the level of B vitamins fed are given in a previous paper (Nielsen and Elvehjem, '41). The individual components of the ration were assayed by the Lactobacillus casei method of Shull, Hutchings and Peterson ('42). The purified ration contained 63.1% less biotin than the unpurified ration, and on a 10 gm. food intake per day would supply only 0.003 µg. of biotin.

Two rats were maintained on this purified ration for 12 weeks and failed to show any symptoms of a biotin deficiency. For comparison purposes a typical rat which received commercial egg white equivalent to 10% of the ration weighed 85 gm. at the end of the seventh week; the fur was unkempt and the back arched; a littermate on the purified ration weighed 181 gm. at the end of the seventh week and appeared normal in all respects. Since the purified ration was furnishing only 0.003 µg. of biotin per day, it appeared that the rats were meeting their requirement by synthesizing this compound.

*Synthesis of biotin.* To obtain some data on the question of synthesis, biotin excretion studies were conducted over a 3-day period during which the rats were placed in metabolism cages in order to collect all excreta. The intake of biotin and all excretion values as determined by the Lactobacillus casei method are expressed in table 2.

Rats no. 1 and no. 2 were placed on the purified ration for 6 weeks and then the excreta were collected. The average synthesis per day was 295 mµg. These same rats were continued on the purified ration for 3 weeks longer and at the end of that period the excreta were collected as before. The average synthesis per day was 358 mµg. (rats 1 A and 2 A in table 2).

In order to study the effects of sulfaguanidine on the synthesis of biotin, rats nos. 3, 4, 5, and 6 were placed on the purified ration containing 0.5% sulfaguanidine. Sulfaguanidine (sulfanilylguanidine), an antibacterial agent which is poorly absorbed from the intestine (Marshall, Bratton, White

TABLE 2

*Synthesis of biotin in the intestinal tract of rats fed various rations.*
*(Biotin values are expressed in mμg.)*

| RAT NO. | WT. | RATION | | | BIOTIN PER GRAM OF FECES | FECES PER DAY | BIOTIN EXCRETION — 3 DAYS | | | BIOTIN SYNTHESIS PER DAY |
| | | Type | Consumed | Biotin content | | | Feces | Urine | Total | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | *gm.* | | *gm* | | | *gm.* | | | | |
| 1 (N)[1] | 160 | B.L.[2] | 35 | 10.5 | 647 | 0.37 | 712 | 89 | 801 | 263 |
| 2 (N) | 112 | B.L. | 38 | 11.4 | 532 | 0.53 | 850 | 144 | 994 | 328 |
| 1A (N) | 219 | B.L. | 33 | 9.9 | 919 | 0.37 | 1010 | 59 | 1069 | 353 |
| 2A (N) | 162 | B.L. | 39 | 11.7 | 509 | 0.67 | 1020 | 82 | 1102 | 363 |
| 3 (N) | 61 | S.G. | 11 | 3.3 | 234 | 0.50 | 234 | 25 | 259 | 128 |
| 4 (N) | 77 | S.G. | 1 | 0.3 | 483 | 0.18 | 85 | 36 | 121 | 121 |
| 5 (N) | 75 | S.G. | 19 | 5.7 | 105 | 0.23 | 74 | 28 | 102 | 32 |
| 6 (N) | 69 | S.G. | 19 | 5.7 | 451 | 0.17 | 225 | 86 | 311 | 102 |
| 7 (S.E.) | 120 | P.A.L. | 23 | 18.8 | 678 | 0.30 | 610 | 130 | 740 | 240 |
| 8 (N) | 83 | P.A.L. | 16 | 13.1 | 625 | 0.20 | 375 | 102 | 477 | 155 |
| 9 (S.E.) | 59 | P.A.L. | 11 | 9.0 | 1603 | 0.13 | 642 | 57 | 699 | 230 |
| 10 (N) | 142 | P.A.L. | 28 | 23 | 1117 | 0.28 | 950 | 136 | 1086 | 354 |
| 11 (P) | 55 | R.L. | 8 | 26.9 | 2480 | 0.07 | 489 | | 489 | 154 |
| 12 (S.E. + P.) | 65 | R.L. | 12 | 40.4 | 1280 | 0.20 | 768 | | 768 | 243 |
| 13 (S.E.) | 88 | R.L. | 19 | 64.0 | 801 | 0.10 | 240 | | 240 | 59 |
| 14 (N) | 163 | Stock | 32 | 2960.0 | 1190 | 2.37 | 8450 | 390 | 8840 | 1960 |

[1] Symptoms: N — normal; S.E. — spectacle eye; P — paralysis.

[2] Rations: B.L. — biotin low; S.G. — sulfaguanidine; P.A.L. — pantothenic acid low; R.L. — riboflavin-low.

and Litchfield, '40), was included in this ration in the hope of reducing the number of intestinal bacteria. A 0.5% level was fed, since previous work (Black, McKibbin and Elvehjem, '41) had indicated that this level was not toxic to the rat, but did reduce the number of intestinal organisms. Excreta were collected at the end of the fifth week from rat no. 3, but unfortunately the animal lived only 2 days after the collection began. The synthesis of biotin was 128 mµg. per day. Rat no. 4 was put into the metabolism cage but died after 20 hours; synthesis for this period was 121 mµg.[4] The excretion of rats nos. 5 and 6, collected for 3 days at the end of the third week, showed synthesis of 32 mµg. and 102 mµg. per day, respectively. Sulfaguanidine tested at a level of 600 µg. per tube had no effect on the assay. Rats no. 5 and no. 6 failed to respond to the injection of 1 µg. of biotin per day over a 7-day period. This finding is not surprising since we were dealing with a complicated deficiency. These animals were deficient not only in biotin but undoubtedly in vitamin K[4] as well. Possibly additional factors elaborated by bacterial synthesis were lacking.

Rats nos. 7, 8, 9 and 10 were maintained on a basal ration deficient in pantothenic acid for a period of 6–8 weeks before the feces were collected. There appeared to be a slight decrease in the total synthesis in the case of rats nos. 8 and 9 as compared with the rats on the biotin-low ration. The intake and amount synthesized are given in table 2.

Rats nos. 11, 12 and 13 were maintained on a riboflavin-low ration for 6–8 weeks. The urine was not collected, since at this time we believed that the amount of biotin found in the urine was insignificant as compared to the total synthesis of this vitamin. There appeared to be a noticeable decrease in the amount of biotin synthesized. The intake and synthesis are given in table 2.

In order to find a value for this synthesis on a natural diet a stock animal (no. 14), comparable to the animals used in the biotin-low ration, was placed in a metabolism cage. The

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[4] Rats on a similar ration were found to be deficient in vitamin K as observed by S. Black of this laboratory.

intake of biotin was very high as the stock ration was fed throughout the test period. The biotin content of the urine was fairly high and the total synthesis was 1960 mμg. per day as shown in table 2. This figure is five times the value obtained on the biotin-low ration.

Three animals receiving the basal ration with 10% of commercial egg white in place of the casein were studied. The total synthesis per day was 1315, 1410 and 1379 mμg. or approximately four times the value obtained on the biotin-low ration. Judging from these values it appears that if there is a means of binding synthesized biotin in the intestinal tract, the bacteria will produce an additional amount.

Since there is synthesis of riboflavin in voided feces of chicks (Lamoreux and Schumacher, '40), the synthesis of biotin in voided feces was studied. Feces were removed from the colon and dried in an oven at 95°C. for 24 hours to destroy bacteria. A 1-day collection of feces from the same animal was allowed to stand in the animal room for 2 days and then dried as described above. The biotin content of feces from rats on different rations was as follows: the feces of a riboflavin-deficient animal as passed contained 706 mμg., and after standing at room temperature for 2 days 949 mμg., or an increase in biotin of 34.4%. The feces of a pantothenic acid-deficient animal under the same conditions contained 843 mμg. and 769 mμg., respectively, or a decrease in biotin of 8.8%. A stock colony animal's feces as passed gave a value of 1055 mμg., but on standing increased to 1635 mμg., a gain of 54.9%. The large increase in this case can probably be attributed to the favorable substrate for bacterial growth afforded by the indigestible material in the stock animal's feces. The additional synthesis of biotin in the experimental rations was not of such a magnitude as to account for the biotin content of the feces as voided.

### SUMMARY

1. Lactobacillus casei and the rat cannot utilize biotin that has been oxidized by mild treatment with hydrogen peroxide (0.3% for 24 hours at room temperature). However, yeast

does utilize biotin treated in this manner. In the assay of biological materials for biotin oxidizing conditions should be avoided in the preparation of the sample.

2. Biotin syntheses varied with different rations. It was highest on the stock ration (1960 mμg. per day per rat) and very low on a riboflavin deficient ration (152). When the sulfaguanidine was added to a biotin-low ration, the lowest value (80) was reached.

3. The preparation of a biotin-low ration has been described.

4. The synthesis of biotin in voided feces has been studied.

## LITERATURE CITED

BIRCH, T. W., AND P. GYÖRGY 1939 Physicochemical properties of the factor (vitamin H) curative of egg white injury. J. Biol. Chem., vol. 131, p. 761.

BLACK, S., J. M. McKIBBIN AND C. A. ELVEHJEM 1941 Use of sulfaguanidine in nutrition experiments. Proc. Soc. Exp. Biol. and Med., vol. 47, p. 308.

BOAS, M. A. 1927 The effect of desiccation upon the nutritive properties of egg white. Biochem. J., vol. 21, p. 712.

BROWN, G. B., AND V. DU VIGNAUD 1941 The effect of certain reagents on the activity of biotin. J. Biol. Chem., vol. 141, p. 85.

FINDLAY, C. M., AND R. O. STERN 1929 A syndrome in the rat resembling pink disease in man. Arch. Dis. Childhood, vol. 4, p. 1.

GAVIN, G., AND E. W. McHENRY 1941 The effects of biotin upon fat synthesis and metabolism. J. Biol. Chem., vol. 141, p. 619.

GYÖRGY, P. 1939 The curative factor (vitamin H) for egg white injury, with particular reference to its presence in different foodstuffs and in yeast. J. Biol. Chem., vol. 131, p. 733.

HEGSTED, D. M., R. C. MILLS, G. M. BRIGGS, C. A. ELVEHJEM AND E. B. HART 1942 Biotin in chick nutrition. J. Nutrition, vol. 23, p. 175.

HOFMANN, K., D. B. MELVILLE AND V. DU VIGNEAUD 1941 Characterization of the functional groups of biotin. J. Biol. Chem., vol. 141, p. 207.

KÖGL, F., AND B. TÖNNIS 1936 Über das Bios-Problem Darstellung von Krystallisiertem Biotin aus Eigelb. Z. physiol. Chem., vol. 242, p. 43.

LAMOREUX, W. F., AND A. E. SCHUMACHER 1940 Is riboflavin synthesized in the feces of fowl. Poul. Sci., vol. 19, p. 418.

MARSHALL, E. K., JR., A. C. BRATTON, H. J. WHITE AND J. T. LITCHFIELD, JR. 1940 Sulfanilylguanidine: A chemotherapeutic agent for intestinal infection. Johns Hopkins Hosp., vol. 67, p. 163.

NIELSEN, E., AND C. A. ELVEHJEM 1941 Cure of spectacle eye condition in rats with biotin concentrates. Proc. Soc. Exp. Biol. and Med., vol. 48, p. 349.

OLESON, J. J., C. A. ELVEHJEM AND E. B. HART 1940 Spectacle eye condition in rats. Proc. Soc. Exp. Biol. and Med., vol. 43, p. 161.

PARSONS, H. T. 1931 The physiological effects of diets rich in egg white. J. Biol. Chem., vol. 90, p. 351.

PARSONS, H. T., AND E. KELLY 1933 The character of the dermatitis producing factor in dietary egg white as shown by certain chemical treatments. J. Biol. Chem., vol. 100, p. 645.

PATRICK, H., R. V. BOUCHER, R. A. DUTCHER AND H. C. KNANDEL 1941 Biotin and prevention of dermatitis in turkey poults. Proc. Soc. Exp. Biol. and Med., vol. 48, p. 456.

PAVCEK, P. L., AND H. M. BAUM 1941 Inositol and spectacled eye in rats. Science, vol. 93, p. 502.

ROBBINS, W. J., AND VIRGENE KAVANAGH 1941 Plant growth substances. Ann. Review of Biochem., vol. 10, p. 499.

SHULL, G. M., B. L. HUTCHINGS AND W. H. PETERSON 1942 A microbiological assay for biotin. J. Biol. Chem., vol. 142, p. 913.

SNELL, E. E., R. E. EAKIN AND R. J. WILLIAMS 1940 A quantitative test for biotin and observations regarding its occurrence and properties. J. Am. Chem. Soc., vol. 62, p. 175.

SYDENSTRICKER, U. P., S. A. SINGAL, A. P. BRIGGS, N. M. DE VAUGHN AND H. ISBELL 1942 Observations on the ''egg white injury'' in man. J. Am. Med. Assoc., vol. 118, p. 1199.

DU VIGNEAUD, V., D. B. MELVILLE, P. GYÖRGY AND C. ROSE 1940 On the identity of vitamin H with biotin. Science, vol. 92, p. 62.

WEST, P. M., AND W. H. WOGLAM 1941 The biotin content of tumors and other tissues. Science, vol. 93, p. 525.

# PERIODIC ADMINISTRATION OF ANTERIOR PITUITARY EXTRACT AS AFFECTING THE METABOLISM OF RATS ON DIETS OF DIFFERENT COMPOSITION [1]

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In the presence of essential accessory food factors, the preferential oxidation or anabolism of protein, carbohydrate and fat within an animal is determined largely by the relative availability of these nutrients at the moment, and by the relative activity of various endocrine secretions in the body. In the latter capacity the secretions of the anterior pituitary undoubtedly play a dominant role, the exact nature of which is, at present, little understood.

As one approach to the study of this problem the present investigation deals with the effects of 3-day pituitary treatments at 2-week intervals on the disposition of the nutrient energy of a balanced diet and of diets characterized by excess of protein, carbohydrate or fat. Also the results are of interest in comparison with the effects of extended daily treatment with A.P.E. (anterior pituitary extract) as reported in previous papers (Voris, Kriss, Marcy and Bowman, '42 a, b) from this Institute.

Since no corresponding investigation has been reported, so far as the present authors are aware, reference to pertinent literature will be made only in the discussion of the results.

## EXPERIMENTAL

Six litter-mate pairs of male albino rats from commercial stock,[2] weighing approximately 100 gm. at the start of the program, were used as experimental subjects. They were kept in individual metabolism cages and allowed tap water ad libitum.

One rat of each pair was injected periodically with a saline anterior pituitary extract (A.P.E.), while the other, serving as the control, was treated similarly with 1% sodium chloride solution. In all other respects both rats of a pair were treated identically.

After a preliminary period of 8 to 10 days during which the limit of voluntary food consumption was determined and the rats gained about 30 gm., they were subjected during a period of 8 weeks to the following treatments in order of sequence: (1) fasting, (2) normal stock [3] diet, (3) carbohydrate-rich diet, (4) protein-rich diet, (5) fat-rich diet, (6) fasting. During the first five treatments respiratory metabolism was measured and urine was collected. In the final fasting test the respiration measurement was omitted and urinary N only was determined as a check on the initial test. Intraperitoneal injections of the A.P.E. were given in 1 ml. portions 2 days prior to, and on the day of, the respiration measurements.

In the fasting period the rats were given food on the day of the first injection; at the time of the second injection food was withdrawn. After 24 hours of fast, and immediately after the third injection, respiration tests were started and continued for 6 hours. Urine was collected for 48 hours following the respiration measurement.

In the feeding periods, each of which lasted 2 weeks, the rats were fed twice daily and trained to eat promptly. The food intake was standardized at 10 gm. daily (5 gm. twice a day). This method of feeding assured experimental uniformity and convenience. At the end of 10 days' preliminary

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[2] Sunny Hill Rat Farm, Clinton, Maryland.
[3] Purina dog chow.

feeding, the first daily injection of A.P.E. was given in the morning, and urine collection was started in the afternoon and continued for 72 hours. After the third injection, and soon after the morning meal was consumed, the respiration measurement was started.

In the preparation of the diets, the basal stock diet was used as a component of the mixed diets. The carbohydrate and protein-rich diets were composed of equal portions by weight of the basal diet and dextrin [4] or beef-muscle protein.[5] The fat-rich diet was composed of the basal diet and hydrogenated vegetable oil [6] in proportions of three to two by weight. A summary of the gross composition of each diet on the dry basis is given in table 1.

TABLE 1

*Gross composition of diets on dry basis.*

|  | STOCK | CARBOHYDRATE RICH | PROTEIN RICH | FAT RICH |
|---|---|---|---|---|
|  | % | % | % | % |
| Carbohydrate | 55.0 | 77.5 | 27.5 | 22.0 |
| Protein | 27.0 | 13.5 | 63.5 | 10.8 |
| Fat | 9.0 | 4.5 | 4.5 | 63.6 |
| Ash | 9.0 | 4.5 | 4.5 | 3.6 |

At the start of each experimental period fresh anterior pituitary extract was prepared from frozen anterior pituitary lobes of cattle.[7] The methods of preparation and preservation have already been described (Voris et al., '42 a). Each milliliter of extract contained 1.5 mg. N.

Respiratory metabolism measurements were made for 6 hours by the Haldane open-circuit gravimetric technic. The apparatus was similar to that described by Forbes, Kriss and Miller ('34) with modifications reported by Kriss ('38). The total $O_2$ and $CO_2$ for the 6 hours were used in computation of the R. Q.'s. The average hourly $CO_2$, excluding the first hour and other hours in which activity was excessive, was used for

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[4] Amidex, Corn Products Refining Co., New York.

[5, 7] Wilson Laboratories, Chicago.

[6] Crisco.

computation of heat production on the basis of $O_2$ consumption values derived therefrom. The gaseous exchange and the heat of protein metabolism were computed on the basis that 1 gm. of urinary N is equivalent to 4.75 liters $CO_2$, 5.96 liters $O_2$ and 26.71 Calories (Kriss and Voris, '37).

Nitrogen was determined in the individual urine samples collected from each rat during the fasting and feeding periods. Carbon and nitrogen were determined in composited urine samples from the feeding periods. Periodic tests were made for acetonuria.

RESULTS

### Urinary N and C

The effects of the A.P.E. on the excretion of urinary N and C are shown in table 2. The fasting results and those on the stock diet were used previously (Voris et al., '42 b) for comparison with those obtained after extended treatment with the extract.

In all periods the urinary N excretion was reduced in the rats treated with the A.P.E. The differences were most significant with the rats on the stock and the protein-rich diets. In the first fasting period two treated rats showed responses opposite to the average, and again, in the second fasting test, the same two rats did not conform to pattern. The divergence of these two rats accounts for the high standard error of the average difference in the fasting urinary N.

It is of interest that the urinary N values relative to the intakes were quite similar for the stock and the carbohydrate-rich diets and for the protein and fat-rich diets, but the values in the first two periods were notably smaller than in the last two.

In the rats treated with the A.P.E. the urinary C excretion was decreased in each feeding period excepting that of the carbohydrate-rich diet. In all feeding periods the ratio of urinary C to N was increased. The decrements in urinary C and N were related in the proportion of 0.4:1, indicating de-

creased secretion of urea (C/N = 0.43). The C/N values for the carbohydrate-rich diet were higher than those on other dietary treatments, indicating a decreased proportion of urea to creatinine (C/N = 1.14) and uric acid (C/N = 1.07).

The absence of ketosis was evident both from the C/N values and the consistently negative tests for acetonuria.

TABLE 2

*Daily excretion of urinary nitrogen and carbon in rats as affected by limited treatment with anterior pituitary extract.*

| TREATMENT | NO. OF RATS | N INTAKE | URINARY N | | URINARY C | C TO N RATIO |
|---|---|---|---|---|---|---|
| | | | Amount | Per cent of intake | | |
| | | *mg* | *mg* [1] | | *mg* | |
| Fasting | | | | | | |
| Treated | 6 | | 118 ± 16 | | | |
| Control | 6 | | 147 ± 18 | | | |
| Difference | | | 29 ± 24 | | | |
| Stock diet | | | | | | |
| Treated | 6 | 432 | 127 ± 3 | 29.4 | 113 | 0.89 |
| Control | 6 | 432 | 166 ± 5 | 38.4 | 130 | 0.78 |
| Difference | | | 39 ± 5 | 9.0 | 17 | 0.44 |
| Carbohydrate-rich | | | | | | |
| Treated | 5 | 216 | 70 ± 3 | 32.4 | 73 | 1.04 |
| Control | 5 | 216 | 78 ± 3 | 36.1 | 70 | 0.90 |
| Difference | | | 8 ± 4 | 3.7 | —3 | ... |
| Protein-rich | | | | | | |
| Treated | 6 | 1016 | 548 ± 7 | 53.9 | 331 | 0.60 |
| Control | 6 | 1016 | 628 ± 13 | 61.8 | 360 | 0.57 |
| Difference | | | 80 ± 15 | 7.9 | 29 | 0.36 |
| Fat-rich | | | | | | |
| Treated | 5 | 173 | 94 ± 4 | 54.3 | 84 | 0.89 |
| Control | 5 | 173 | 106 ± 3 | 61.3 | 89 | 0.84 |
| Difference | | | 12 ± 5 | 7.0 | 5 | 0.42 |
| Fasting | | | | | | |
| Treated | 6 | | 88 ± 6 | | | |
| Control | 6 | | 100 ± 3 | | | |
| Difference | | | 12 ± 7 | | | |

[1] ± values represent the standard errors of means or differences.

## Energy metabolism

The effects of the A.P.E. on the respiratory quotients, the total heat production and the nutrient derivation of heat are presented in table 3. For purposes of comparison with the effects of extended treatment data pertaining to fasting and the stock diet (Voris et al., '42 b) are also given.

Under all treatments, fasting or feeding, the total heat production was increased in the rats treated with the A.P.E. In each case, the extra heat was produced from non-protein nutrients, and the heat derived from protein was decreased. Thus, the increase in total heat production of the treated rats resulted from an accelerated oxidation of non-protein nutrients, which was greater in magnitude than a simultaneous diminution in protein katabolism.

In the fasting period the control rats produced 21%, and the treated rats 14%, of their total heat from protein. On the stock diet the proportionate derivation of heat from protein and non-protein nutrients was almost identical with that of fast. During the feeding of the stock diet the control rats produced 78%, and the treated rats 86%, of their total heat from carbohydrate, while in the fasting period the same energy quotas were derived from fat. The non-protein respiratory quotients indicated that the control rats on the stock diet were synthesizing fat while the treated rats were oxidizing extra carbohydrate. Computation of the glucose equivalent indicated that the treated rats were oxidizing about 1.5 times as much extra carbohydrate as the control rats were synthesizing into fat. The difference in the R. Q.'s was not highly significant (odds 20 to 1 against difference occurring by chance alone) so that the computation does not merit emphasis if considered alone. However, the same relationship was found on the carbohydrate-rich diet for which the difference in R. Q.'s was of the same magnitude as that for the stock diet but statistically more highly significant.

In the feeding of the carbohydrate and the fat-rich diets the protein katabolism was diminished considerably. This resulted

TABLE 3

The effect of periodic injection of anterior pituitary extract on the total heat production and the heat derived from protein, carbohydrate and fat.

| TREATMENT | NUMBER OF RATS | NON PROTEIN R Q | TOTAL HEAT PRODUCTION | HEAT DERIVED FROM | | | Fat synthesis |
|---|---|---|---|---|---|---|---|
| | | | | Protein | Carbohydrate | Fat | |
| | | | Cal/hr | % | % | % | % |
| Initial fast | | | | | | | |
| Treated | 4 | 0.70 ± 0.01 | 911 | 14.4 | 0 | 85.6 | 0 |
| Control | 4 | 0.71 ± 0.01 | 775 | 21.0 | 0.8 | 78.2 | 1.1 |
| Stock diet | | | | | | | |
| Treated | 4 | 1.00 ± 0.03 | 1025 | 14.2 | 85.8 | 0 | 0 |
| Control | 4 | 1.05 ± 0.03 | 878 | 20.8 | 78.1 | 0 | 1.1 |
| Carbohydrate-rich diet | | | | | | | |
| Treated | 4 | 1.09 ± 0.01 | 1111 | 6.5 | 91.8 | 0 | 1.7 |
| Control | 4 | 1.14 ± 0.01 | 972 | 5.7 | 98.6 | 0 | 2.7 |
| Protein-rich diet | | | | | | | |
| Treated | 4 | 0.91 ± 0.03 | 1224 | 49.4 | 34.9 | 15.7 | 0 |
| Control | 4 | 1.02 ± 0.03 | 1114 | 61.8 | 37.9 | 0 | 0.3 |
| Fat rich diet | | | | | | | |
| Treated | 6 | 0.79 ± 0.01 | 1397 | 7.8 | 28.1 | 64.1 | 0 |
| Control | 6 | 0.79 ± 0.00 | 1239 | 9.7 | 28.6 | 61.7 | 0 |

from the decreased intake of dietary protein rather than from a sparing of protein katabolism by the non-protein nutrients (table 2). However, the protein sparing effect of the A.P.E. was still in evidence, and the difference, although not as conspicuous in these periods as in the others, was highly significant statistically.

The protein sparing effect of the A.P.E. was naturally most conspicuous in the feeding of the protein-rich diet. In this period the control rats produced 62%, and the treated rats 49%, of their total heat from protein. The heat of carbohydrate oxidation was about equal for the control and the treated rats, so that the extra heat produced by the latter was derived mostly from fat.

TABLE 4

*Average weights and gains of rats during 2 weeks on the various diets. Treated rats (T) were injected with A.P.E. for 3 days prior to each period.*

*C = control pairmates.*

| | TYPE OF DIET | | | | | | | | OVERALL | |
| | Stock | | Carbohydrate-rich | | Protein-rich | | Fat-rich | | | |
| | T | C | T | C | T | C | T | C | T | C |
|---|---|---|---|---|---|---|---|---|---|---|
| Final wt., gm. | 150 | 151 | 170 | 176 | 214 | 216 | 236 | 240 | 236 | 240 |
| Initial wt., gm. | 106 | 106 | 150 | 151 | 170 | 176 | 214 | 216 | 106 | 106 |
| Gain | 44 | 45 | 20 | 25 | 44 | 40 | 22 | 24 | 130 | 134 |

On the fat-rich diet the extra heat of the treated rats was derived from both carbohydrate and fat, but the latter contributed about four times as much energy as the former in the increased metabolism.

The weights and gains of the rats during the various dietary treatments are presented in table 4. The A.P.E. was administered for 3 days prior to the start of each dietary treatment, this being also the last 3 days of the prevailing treatment. With this short injection period the growth principle of the A.P.E. evidently did not affect body weight (Voris et al., '42 a), so that the results of table 4 are significant only insofar as they represent the carry-over of the A.P.E. as affecting

the compromise between the conservation of protein and the utilization of extra dietary energy for the increased metabolism. The rats treated with the A.P.E. showed excess gain over the controls only on the protein-rich diet. The difference was not statistically significant, but assumed a significance in comparison with the results on the carbohydrate and the fat-rich diets in which the weight differences favoring the control rats were statistically significant.

### DISCUSSION

The results presented herein confirm and extend those presented by Voris et al. ('42 b) showing that the saline A.P.E. used in these investigations spared protein but increased the oxidation of non-protein nutrients. Also the conclusion of these authors that the increased heat production of the treated rats was the result of a true stimulation of cellular metabolism, and not the accelerated oxidation of a particular nutrient, is well supported by the present results.

The energy required for the accelerated metabolism was derived from whatever nutrient was most available at the moment. Carbohydrate appeared to be the preferred source of energy for immediate metabolic purposes.

Protein was preferentially katabolized only when the supply exceeded the body capacity for retention. Under such a condition protein may spare the oxidation of carbohydrate or fat in the same way that it is spared when the latter nutrients are in excess.

Fat was oxidized readily when its presence in the diet overbalanced that of carbohydrate and protein, but the oxidation of carbohydrate was not entirely excluded by the plethora of fat as the oxidation of fat was excluded when dietary carbohydrate was sufficient.

These observations merely represent well-recognized metabolic principles: carbohydrate is the preferred source of energy for metabolism; protein is necessary for the maintenance of cellular structure, and incidentally provides a potential source of energy; while fat is conserved under

ordinary conditions as the most efficient form of energy reserve for emergency use.

The principle of the anterior pituitary which depresses carbohydrate oxidation and increases carbohydrate reserves (Fisher, Russell and Cori, '36; Meyer, Wade and Cori, '37; Russell, '38 a) was not in evidence in the A.P.E. used in the present investigation. Russell ('38 b) has shown that the principle is not associated with the growth, adrenotropic, gonadotropic, lactogenic, or thyrotropic factors of the anterior pituitary. Greaves, Freiberg and Johns ('40) have associated the R. Q. reducing factor with the ketogenic principle.

The investigations describing the glycostatic action of the anterior pituitary have dealt with the immediate physiological response of rats to the A.P.E. with respect to a single nutrient (glucose). Under the conditions imposed the animals were in a state of physiological readjustment and the metabolic measurement could well represent the adjustmental processes rather than the regulated processes of normal nutrition. The negative effects obtained after 20 days' treatment with the A.P.E. (Russell, '38 a) may represent the latter condition rather than a refractory reaction to continued treatment.

The standardized feeding technic and the 3-day treatment employed in the present study avoided the immediate adjustmental responses to the A.P.E., as well as protein plethora, and the antagonistic effects encountered with extended treatment (Voris et al., '42 b).

Recently Gaebler and Robinson ('42) have presented further evidence that the growth preparation of the anterior pituitary stimulates the production or mobilization of insulin. However, insulin alone does not induce N storage in normal animals, nor does A.P.E. alone induce N storage in depancreatized dogs receiving insulin. While the appearance of the protein sparing or diabetogenic effects of an A.P.E. may depend on the capacity of the pancreas to produce insulin, it would seem that the effects are less specific than as suggested by Gaebler and Robinson ('42) in view of the results reported in this paper. It seems more probable that all regulatory agents con-

cerned in the maintenance of homeostasis are affected, and that the net result will depend on the capacity of the various organs producing the regulatory agents to meet the demands made upon them.

## SUMMARY

The effects of limited treatment (3 days) of rats with saline anterior pituitary extract have been studied with respect to the disposition of the nutrient energy of a balanced diet, and of diets characterized by excess of protein, carbohydrate and fat. Measurements of the energy metabolism and the secretion of urinary N and C were made under each of the dietary treatments. A standardized feeding method for the treated and the control rats was used to assure experimental uniformity and nutritional adjustment to the diets prior to the metabolism measurements.

Under all dietary conditions the A.P.E. decreased the katabolism of protein, but stimulated the oxidation of nonprotein nutrients. The latter effect overbalanced the former, so that the total heat production was increased. The extra heat resulted from a true stimulation of cellular metabolism, and not from the accelerated oxidation of any particular nutrient.

## LITERATURE CITED

FISHER, R. E., J. A. RUSSELL AND C. F. CORI 1936 Glycogen disappearance and carbohydrate oxidation in hypophysectomized rats. J. Biol. Chem., vol. 115, p. 627.

FORBES, E. B., M. KRISS AND R. C. MILLER 1934 The energy metabolism of the albino rat in relation to the plane of nutrition. J. Nutrition, vol. 8, p. 535.

GAEBLER, O. H., AND A. R. ROBINSON 1942 Effects of the pancreas and the adrenals upon production of nitrogen storage with pituitary preparations. Endocrin., vol. 30, p. 627.

GREAVES, J. D., I. K. FREIBERG AND H. E. JOHNS 1940 Preparation and assay of anterior pituitary fractions rich in ketogenic and respiratory quotient-reducing substances. J. Biol. Chem., vol. 133, p. 243.

KRISS, M. 1938 The specific dynamic effects of proteins when added in different amounts to a maintenance ration. J. Nutrition, vol. 15, p. 565.

KRISS, M., AND L. VORIS 1937 A further contribution to the derivation of factors for computing the gaseous exchange and the heat production in the metabolism of proteins. J. Nutrition, vol. 14, p. 215.

MEYER, H. S., L. J. WADE AND C. F. CORI 1937 Influence of extracts of anterior lobe of pituitary on glucose oxidation and glycogen storage. Proc. Soc. Exp. Biol. Med., vol. 36, p. 346.

RUSSELL, J. A. 1938 a The effects of hypophysectomy and of anterior pituitary extracts on the disposition of fed carbohydrate in rats. Am. J. Physiol., vol. 121, p. 755.

———— 1938 b The anterior pituitary factor which maintains muscle glycogen in fasted hypophysectomized rats. Endocrin., vol. 22, p. 80.

VORIS, L., M. KRISS, L. F. MARCY AND R. S. BOWMAN 1942 a Effects of prolonged daily treatment of normal rats with saline anterior pituitary extract. I. Sexual differences in appetite, growth and organ weights. J. Nutrition, vol. 24, p. 469.

———— 1942 b Effects of prolonged daily treatment of normal rats with saline anterior pituitary extract. II. Protein and energy metabolism. J. Nutrition, vol. 24, p. 481.

# THE METABOLISM OF ARGININE AND LEUCINE WITH SPECIAL REFERENCE TO RESPIRATORY EXCHANGE AND HEAT PRODUCTION [1]

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The purpose of this investigation was to add to the existing determinations of the dynamic effects of amino acids of different groups by the particular procedure employed, that is, by difference between values for heat production from established nutritive regimens, with the metabolism of energy equilibrium as the base value.

The series of such determinations made at this laboratory, including those presented in an earlier paper (Kriss, '41) and those now here reported, represent the following kinds and classes of amino acids: monoamino monocarboxylic acids: glycine, dl-alanine, l-leucine and l-tyrosine; monoamino dicarboxylic acids: d-glutamic acid, l-aspartic acid and its half-amide l-asparagin; diamino monocarboxylic acid: arginine. Among these seven compounds only leucine and arginine, the subjects of the present study, are essential in the nutrition of the rat.

The dynamic effect of any amino acid, by whatever method determined, is in all probability affected by the other nutrients with which it is metabolized, since it would never be metabolized alone. This fact should be kept in mind, therefore, in

comparing dynamic effects determined by procedures implying the metabolism of fast as the base value with determinations made, as in the present investigation, with reference to the metabolism of nutritive equilibrium.

It is the authors' understanding that the advantage of employing the heat production of maintenance as the base value is that dynamic effects so determined represent conditions of practical nutrition, while dynamic effects determined with the heat production of any submaintenance nutritive status are invalidated by an assumed dynamic effect of the body nutrients katabolized — a point of view that has been stressed in several papers from this Institute since 1928 (Forbes and Swift, '41 a, '41 b; Kriss, '41).

In the present investigation the conditions were such that the dynamic effects of the amino acids, as observed, applied to the use of these acids for heat production alone since the amino acids were almost completely absorbed, and after absorption were completely eliminated in the urine.

### DETAILS OF EXPERIMENTATION

This experiment was conducted during the months of October and November, 1940.

The subjects were twelve, growing, male, albino rats, six of which (nos. 61 to 66, inclusive) were used to determine the dynamic effect of arginine monohydrochloride, and six others (nos. 67 to 72, inclusive) for a similar study of leucine

The final results for both groups, however, represent only five individuals, since rat no. 65 was eliminated because of diarrhea, and rat no. 70 was accidentally suffocated during the final respiration experiment.

The basal diet was 9 gm. per day of a nutritively complete commercial feed [a] 90% and butterfat 10%, the rats being fed twice a day, at 8:00 A.M. and 8:00 P.M. The butterfat was included for its contribution to the palatability of the diet.

The average initial body weights of the arginine and the leucine rats were 220 and 200.5 gm., respectively, and their

[a] Purina dog chow.

average weights before the final period of fast were 238 and 226.7 gm., respectively. The basal diet, therefore, provided slightly more than maintenance requirements, as intended.

The quantities of amino acids fed daily as supplements were 1.14 gm. of arginine monohydrochloride and 1.18 gm. of l-leucine, these quantities providing 10.7% and 15.2%, respectively, of the gross energy, and 50.5% and 29.4%, respectively, of the nitrogen of the entire diets in which they were fed.

Dynamic effects were determined by difference between the heat production from the basal diet and from the same plus an amino acid.

Respiration measurements, by the open-circuit Haldane procedure, began at 8:30 A.M. and continued for 7 hours.

A representative schedule of experimentation is that of rat no. 61, as follows: basal diet, October 14th–October 28th; collection of excreta, October 21st–October 28th; respiration measurement, October 28th; supplemented diet, basal plus arginine, October 28th–November 6th; collection of excreta, November 1st–November 6th; respiration measurement, November 6th; basal diet, November 6th–November 12th; respiration measurement, November 12th; fast, November 18th–19th; respiration measurement November 19th.

The alimentary fill was determined on all rats on November 27th; and the urines and the feces of the similarly treated rats were composited for analysis, the elimination of excretory constituents of the individual animals being computed from the individual quantities of excreta eliminated and the analyses of the composite samples.

The metabolism of protein and of amino acids was accounted for in the computation of the heat production in the feeding periods on the basis of the urinary nitrogen excretions and the respiration and energy factors reported by Kriss ('39) and Kriss and Marcy ('40).

The usual practice at this laboratory of computing the heat production, for comparative purposes, to a basis of uniform empty body weight was continued in the present study.

The analysis of the diets is given in table 1, and that of the urines in table 2; the data for daily income, feces, urine and metabolizability of the amino acids are set forth in table 3.

These data signify that the energy and the carbon of the arginine monohydrochloride were not so well absorbed as those of the leucine; that the energy and the carbon intakes in arginine monohydrochloride were more largely eliminated in the urine than were those in leucine; that approximately all of the nitrogen of the arginine monohydrochloride and of the leucine was absorbed, metabolized, and eliminated in the urine; and that of the energy of the arginine monohydrochloride and the leucine, 64.8% and 90.0%, respectively, was metabolized.

TABLE 1

*Analysis of basal ration and amino acid supplements.*

| MATERIAL | MOISTURE | NITROGEN | CARBON | ENERGY |
|---|---|---|---|---|
| | % | % | % | cal./gm |
| Basal ration | 8.39 | 3.25 | 43.55 | 4650 |
| Arginine monohydrochloride | 0.10 | 26.21 | 33.92 | 4416 |
| l-Leucine | 0.11 | 10.30 | 53.90 | 6369 |

Since protein utilization was not increased by the ingestion of these two essential amino acids, it is clear that the protein requirements of the experimental subjects were satisfied by the basal diet. The heat increments of the amino acids as observed, therefore, were unaffected by growth.

The basis on which the factors were determined for computing the respiratory exchange and the heat production in the metabolism of arginine and leucine is set forth in table 4. These values refer to moisture-free leucine, and to the pure arginine in the arginine monohydrochloride.

Values for the hourly heat production of the rats per 200 gm. of empty body weight, and for the increases in heat production due to the amino acids, on the absolute basis and

TABLE 2

*Urinary analysis.*

| RAT NO. | BASAL RATION | | | | BASAL RATION PLUS 1.14 GM ARGININE MONOHYDROCHLORIDE | | | | DIFFERENCES DUE TO AMINO ACIDS | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Nitrogen per day | Carbon per day | C:N ratio | Energy per day | Nitrogen per day | Carbon per day | C:N ratio | Energy per day | Nitrogen per day | Carbon per day | C:N ratio | Energy per day | ENERGY:N RATIO |
| | *mg.* | *mg.* | | *cal.* | *mg.* | *mg.* | | *cal.* | *mg.* | *mg.* | | *cal.* | |
| 61 | 138 | 128 | 0.93 | | 426 | 229 | 0.54 | | 288 | 101 | 0.35 | | |
| 62 | 130 | 127 | 0.98 | | 431 | 235 | 0.55 | | 301 | 108 | 0.36 | | |
| 63 | 128 | 124 | 0.97 | | 426 | 237 | 0.56 | | 298 | 113 | 0.38 | | |
| 64 | 132 | 117 | 0.89 | | 414 | 231 | 0.56 | | 282 | 114 | 0.40 | | |
| 66 | 138 | 131 | 0.95 | | 440 | 245 | 0.56 | | 302 | 114 | 0.38 | | |
| Average | 133 | 125 | 0.94 | 1321 | 427 | 235 | 0.55 | 2725 | 294 | 110 | 0.37 | 1404 | 4.78 |
| BASAL RATION PLUS 1 18 GM LEUCINE | | | | | | | | | | | | | |
| 67 | 140 | 123 | 0.88 | | 268 | 178 | 0.66 | | 128 | 55 | 0.43 | | |
| 68 | 136 | 123 | 0.90 | | 268 | 184 | 0.69 | | 132 | 61 | 0.46 | | |
| 69 | 133 | 124 | 0.93 | | 257 | 185 | 0.72 | | 124 | 61 | 0.49 | | |
| 70 | 135 | 129 | 0.96 | | 248 | 182 | 0.73 | | 113 | 53 | 0.47 | | |
| 71 | 158 | 140 | 0.89 | | 266 | 192 | 0.72 | | 108 | 52 | 0.48 | | |
| 72 | 153 | 132 | 0.86 | | 262 | 185 | 0.71 | | 109 | 53 | 0.49 | | |
| Average | 143 | 129 | 0.90 | 1371 | 262 | 184 | 0.70 | 2119 | 119 | 56 | 0.47 | 748 | 6.29 |

as related to the metabolizable energy of the amino acids, are given in table 5.

On either the former or the latter basis these amino acids unquestionably manifested dynamic effects, but these effects were more variable, as among the individual subjects, than were similar values derived in the earlier studies of similar character.

TABLE 3

*Metabolizability of amino acids.*

|  | ARGININE MONO-HYDROCHLORIDE 1.14 GM. | LEUCINE 1.18 GM. |
|---|---|---|
| Income |  |  |
| Energy, cal. | 5034 | 7515 |
| Carbon, mg. | 387 | 636 |
| Nitrogen, mg. | 299 | 121 |
| Outgo |  |  |
| Feces |  |  |
| Energy, cal. | 369 | – |
| Carbon, mg. | 23 | – |
| Nitrogen, mg. | 5 | 2 |
| Urine |  |  |
| Energy, cal. | 1404 | 748 |
| Carbon, mg. | 110 | 56 |
| Total nitrogen, mg. | 294 | 119 |
| Unmetabolized { mg.[1] | 0 | 1 |
| amino acid N { mg.[2] | 0 | 2 |
| Metabolized { mg.[1] | 294 | 118 |
| amino acid N { mg.[2] | 294 | 117 |
| { %[1] | 100 | 99 |
| { %[2] | 100 | 98 |
| Metabolizable |  |  |
| Energy, cal. | 3261 | 6767 |
| Carbon, mg. | 254 | 580 |
| Energy, % | 64.8 | 90.0 |
| Carbon, % | 65.6 | 91.2 |

[1] Computed on the basis of C : N ratio.

[2] Computed on the basis of energy : N ratio.

TABLE 4

*Determination of factors for computing the respiratory exchange and the heat production in the metabolism of arginine and leucine.*

| | ARGININE | LEUCINE |
|---|---|---|
| Intake, computed per 100 gm. of pure amino acids | | |
| Energy, Cal. | 534.6 | 637.6 |
| Nitrogen, gm. | 32.16 | 10.68 |
| Carbon, gm. | 41.36 | 54.94 |
| Hydrogen, gm. | 8.10 | 9.99 |
| Oxygen, gm. | 18.37 | 24.40 |
| Outgo | | |
| Feces | | |
| Energy, Cal. | 8.8 | |
| Nitrogen, gm. | 0.53 | |
| Carbon, gm. | 0.68 | |
| Hydrogen, gm. | 0.13 | |
| Oxygen, gm. | 0.30 | |
| Urine | | |
| Energy, Cal. | 151.0 | 67.1 |
| Total nitrogen, gm. | 31.63 | 10.68 |
| Unmetabolized amino acid N, gm. | 0.00 | 0.13 |
| Metabolized N, gm. | 31.63 [1] | 10.55 |
| Carbon, gm. | 11.83 | 5.03 |
| Hydrogen, gm. | 4.80 | 1.64 |
| Oxygen, gm. | 15 80 | 6.33 |
| Metabolized | | |
| Energy, Cal. | 374.8 | 570.5 |
| Carbon, gm. | 28.85 | 49.91 |
| Hydrogen, gm. | 3.17 | 8.35 |
| Oxygen, gm. | 2.27 | 18.07 |
| Intramolecular H O | | |
| Hydrogen, gm. | 0.28 | 2.26 |
| Oxygen, gm. | 2.27 | 18.07 |
| Carbon oxidized to $CO_2$, gm. | 28.85 | 49.91 |
| Hydrogen oxidized to $H_2O$, gm. | 2.89 | 6.09 |
| $CO_2$ produced, gm. | 105.78 | 183.00 |
| $O_2$ required to oxidize C, gm. | 76.93 | 133.09 |
| $O_2$ required to oxidize H, gm. | 23.12 | 48.72 |
| Total $O_2$ required, gm. | 100.05 | 181.81 |
| Respiratory quotient | 0.77 | 0.73 |
| Liters $O_2$ required per gram urinary N | 2.21 | 11.91 |
| Liters $CO_2$ produced per gram urinary N | 1.70 | 8.73 |
| Calories metabolized per gram urinary N | 11.8 | 53.4 |
| Calories per liter $O_2$ | 5.35 | 4.48 |
| Liters $O_2$ required per gram N of amino acid metabolized | 2.21 | 12.06 |
| Liters $CO_2$ produced per gram N of amino acid metabolized | 1.70 | 8.84 |
| Calories metabolized per gram N of amino acid metabolized | 11.8 | 54.1 |

[1] Of the total urinary nitrogen 3.98 gm. were calculated as ammonia and 27.65 gm. as urea.

With rat no. 62 the arginine monohydrochloride incited heat production to an extent in excess of its own metabolizable energy.

With the remaining rats of this group the dynamic effect of arginine and leucine varied between 45.8 and 77.9%, and 19.2 and 49.3%, respectively, of their metabolizable energy. In neither case does it seem justified to assume that the true value is a physiological constant. The dynamic effects, therefore, are not averaged.

TABLE 5

*Hourly heat production of rats per 200 gm of empty weight and increase in heat production due to the amino acids*

| RAT NO | BASAL DIET | | | BASAL DIET | INCREASE IN HEAT PRODUCTION DUE TO AMINO ACID | |
| | Initial period | Final period | Average | | Total | Per cent of metabolizable energy |
| --- | --- | --- | --- | --- | --- | --- |
| | cal | cal | cal | + 1 14 gm arginine | cal | |
| 61 | 941 | 995 | 968 | 1081 | 113 | 77.9 |
| 62 | 971 | 1056 | 1014 | 1191 | 177 | 137.2 |
| 63 | 1014 | 957 | 986 | 1054 | 68 | 50 7 |
| 64 | 964 | 954 | 959 | 1040 | 81 | 63 3 |
| 66 | 978 | 999 | 989 | 1054 | 65 | 45 8 |
| Average | 974 | 992 | 983 | 1084 | | |
| | | | | + 1 18 gm leucine | | |
| 67 | 1087 | 948 | 1018 | 1156 | 138 | 49.3 |
| 68 | 944 | 951 | 948 | 1025 | 77 | 27 6 |
| 69 | 1068 | 998 | 1033 | 1087 | 54 | 19.2 |
| 71 | 987 | 920 | 954 | 1020 | 66 | 23.2 |
| 72 | 1049 | 969 | 1009 | 1100 | 91 | 31.9 |
| Average | 1027 | 957 | 992 | 1078 | | |

Since the variation among the absolute individual values was similar to that among the values referred to the average metabolizable energy, it is necessary to ascribe this variation primarily to two factors, (1) individual response of the experimental subjects to the associative effect of the basal diet and the amino acid supplements, and (2) the inevitable errors of experimental work, which in these cases were referred, by

the difference method of determination of the heat increments, to the relatively small proportions of these amino acids (10.7% and 15.2%, respectively, of the gross energy of the diets) that the rats were able to take.

The dynamic effects observed were not constant enough to serve as a basis of close comparison with the dynamic effects of other amino acids.

## SUMMARY

With albino rats as subjects, the dynamic effects of arginine monohydrochloride and leucine were determined by super-imposition upon a nutritively complete basal diet.

Virtually all of the nitrogen of these amino acids was absorbed, metabolized, and eliminated in the urine; and of the energy of these two compounds, 64.8% and 90.0%, respectively, was metabolized.

The dynamic effects of arginine and leucine varied between 45.8% and 137.2%, and 19.2% and 49.3%, respectively, of their metabolizable energy. In neither case does it seem justified to assume that the true value is a physiological constant.

### LITERATURE CITED

FORBES, E. B., AND R. W. SWIFT 1941 a  The minimum base value of heat production in animals. Science, vol. 93, p. 623.

FORBES, E. B., AND R. W. SWIFT, WITH COLLABORATION 1941 b  The minimum base value of heat production in animals. A research in the energy metabolism of cattle. Penna. Agr. Expt. Sta., Bul. 415.

KRISS, MAX 1939  A comparative study of the metabolism of certain amino acids, with special reference to respiratory exchange and heat production. J. Nutrition, vol. 17, no. 1, p. 1.

———— 1941  The specific dynamic effects of amino acids and their bearing on the causes of specific dynamic effects of proteins. J. Nutrition, vol. 21, no. 3, p. 257.

KRISS, MAX, AND LAWSON F. MARCY 1940  The metabolism of tyrosine, aspartic acid and asparagine, with special reference to respiratory exchange and heat production. J. Nutrition, vol. 19, no. 3, p. 297.

# AN EVALUATION OF THE NUTRITIONAL STATUS OF A POPULATION GROUP IN MADRID, SPAIN, DURING THE SUMMER OF 1941 [1]

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This communication deals with an attempt to ascertain some facts regarding the nutritional state of a population group in Madrid, Spain, during the summer of 1941. The survey was confined to an industrial suburb of Madrid. It was made on a family basis with the sample drawn in accordance with the geographic distribution of the population, and utilized the combined methods of dietary records, clinical examination, and appropriate laboratory determinations. In all, 106 families composed of 561 individuals were examined between the end of May and the middle of September, 1941.

## METHODS

The dietary records were collected on a family basis by the inventory and purchase method (Bigwood, '39), the consumption of foodstuffs by each family being determined for

7 consecutive days. Each family was visited daily, and the amount of food purchased that day was weighed, together with the refuse, if any, from the previous day. At the same time the cost of each item of food was recorded, furnishing a record of the amount spent for food by the family during the week. This family record was supplemented by the following individual records made by the investigator during the week of the family inquiry: (1) household measurements of the meal by food consumption of one adult in each family for 3 nonconsecutive days, (2) number of servings of each foodstuff eaten during the week by each child under 10 years of age, obtained by questioning and expressed in household measurements, (3) feeding since birth of all infants under 2 years of age. With families who were receiving meals from the relief agency, information was obtained from an official of the agency as to the quantity fed per person of each foodstuff used in preparing the dish served each day.

For the calculation of the dietary constituents, food tables were compiled in an attempt to approximate the content of Spanish foods, in so far as this was possible with available data. Carbohydrate, fat, protein, and caloric values were obtained from the few analyses which have been made of Spanish foodstuffs (Vazquez Sanchez, '32; Puyal and Srta. Torres, '32; Grande Covian, '39) and from European sources (Randoin, '39; Schall, '41). It was necessary to assume from analyses in other countries the values for minerals (Sherman, '37) and vitamins (Booher and Hartzler, '39; Fixsen and Roscoe, '40; Droese and Bramsell, '41). A single value for vitamin content usually had to be selected arbitrarily from a wide range of values offered by analyses in other parts of the world. This is a probable source of error particularly with respect to vitamins A and C, which are known to vary considerably with geographic and seasonal sources. No allowance has been made for losses in cooking; the cooking habits were such that this loss was probably small.

The clinical examination consisted of the usual diagnostic history and physical examination, with special attention

directed to symptoms and signs of deficiency disease and the detection of concomitant conditions which may affect the nutritional status. In addition, the cornea was examined with the slit lamp and biomicroscope for evidence of capillary invasion, roentgenograms were obtained of the hand and lower leg on all children under 10 years of age, and certain anthropometric determinations were made.

About 20 cc. of venous blood was drawn from each subject with precautions against stasis and hemolysis; 4 cc. was oxalated with potassium and ammonium oxalate in suitable proportions and quantity to prevent change in cell size. On this was determined the hemoglobin concentration, erythrocyte count, and hematocrit. The remainder was allowed to clot.

The hemoglobin was determined by the method of Evelyn ('36) for oxyhemoglobin, the readings being made with the photoelectric colorimeter and converted to grams hemoglobin per 100 cc. by using K 2.58. Frequent checks indicated the accuracy of this method under conditions of the survey to be ±0.2 gm. per 100 cc.

The red blood cell counts were made by the usual method using Thoma diluting pipettes, shaking by hand, and counting areas in two counting chambers on each specimen. Repeated duplicate counts indicated the accuracy under survey conditions to be ±150,000 per cubic millimeter.

The hematocrit determinations were made in standard Wintrobe tubes, the packed red cell volumes being red after 1 hour centrifuging at 3500 r.p.m. Duplicate determinations indicated an accuracy of ±0.5 vol. per cent.

Serum protein and serum albumin were determined by the quantitative biuret reaction (Robinson and Hogden, '40). The readings were made on the photoelectric colorimeter with a standard of preserved rabbit serum on which several macro-Kjeldahl nitrogen determinations were made. The separation of albumin and globulin was obtained by salting out with 22% sodium sulphate and filtration.

Serum ascorbic acid content was determined by the titration of a metaphosphoric acid filtrate of the serum with 2,6

dichlorophenolindophenol (Farmer and Abt, '35). The dye
was standardized against fresh solutions of ascorbic acid, and
redistilled water used in all reagents.

Serum carotene and vitamin A were determined by using a
petroleum ether extract of alcohol-treated serum and the
Carr-Price reaction, utilizing the micro-unit of the photo-
electric colorimeter (May et al., '40). The carotene readings
were made with a 420 filter, but a reference curve derived with
this filter for beta-carotene was used as a standard. The
vitamin A content was calculated by using the following
arbitrary constants:

$L_{630}$–0.11 $L_{420}$ to correct for blue color contributed by carotenoids

0.41 to convert $L_{1\,cm.}^{1\%}$ to $E_{1\,cm.}^{1\%}$

2100 to convert $E_{1\,cm.}^{1\%}$ to International Units.

The phosphatase activity and the inorganic phosphorus con-
tent of the sera of children under 6 years of age were deter-
mined by the methods of Bodansky ('33) and Fiske and Sub-
barow ('25), respectively.

### CHARACTERISTICS OF SAMPLE

The district in which the subjects of this study lived, Puente
de Vallecas, is on the outskirts of Madrid, directly continuous
with the city itself and extending out into the arid plain on
which the capital is set. While some of the inhabitants go
out to work by the day in the fields, there was no significant
production of food in the area for home consumption. The
chief industries are the railroad repair shops and the tan-
neries. Most of the subjects were unskilled or semiskilled
laborers or were engaged in small business enterprises.
Thirty-two of the 106 families studied were receiving a sub-
stantial amount of food from the official relief agency, Auxilio
Social. For most of these, this consisted of one serving of thick
soup daily for each member of the family; in a few families
some of the children between the ages of 5 and 12 years were
getting two meals a day in the dining rooms of this agency.

During the period of the survey this agency was feeding about 12,500 persons a day, some 10,600 from the soup kitchens and 1,850 in children's dining rooms.

The census of December, 1940, lists the population of the district at 58,878; this figure is estimated to be about 20% below the actual population level. Therefore the sample represents less than 1% of the total population of the district, and is weighted with a larger proportion of families receiving relief assistance. The sample does conform fairly well to the density of population in the geographic subdivisions of the area.

TABLE 1

*Age and sex distribution of subjects.*

| SEX | AGE IN MONTHS | AGE IN YEARS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2–24 | 2–5 | 6–12 | 13–19 | 20–29 | 30–39 | 40–49 | 50–59 | 60 and over | Total |
| Males | 17 | 30 | 83 | 42 | 14 | 20 | 33 | 10 | — | 249 |
| Females | 24 | 35 | 83 | 40 | 28 | 46 | 36 | 8 | 12 | 312 |
| Total | 41 | 65 | 166 | 82 | 42 | 66 | 69 | 18 | 12 | 561 |

Table 1 presents the distribution by age and sex of the subjects studied. It should be noted that nearly one-half of the total were under 13 years of age and that there were fewer men than women between the ages of 20 and 40. No data were available on the age and sex distribution of the general population.

Inadvertent selection was undoubtedly operating to some extent, as in all surveys of this nature, since only persons who will cooperate in the diet inquiry, clinical examination, and bloodletting can be studied. The incentive offered, 1 kg. of flour or two cans of evaporated milk for each person completing the entire study, might have been expected to bring in families with more limited food supplies. Actually most of the refusals occurred among persons who were unable or unwilling to understand the purpose of the study, and these were more frequent among the poorer families. There were nine additional families on whom the dietary records were

completed who subsequently refused the medical examination; their dietary intake and expenditure for food fall in the range of the 106 families completely studied.

### FINDINGS

The data collected are presented in tables which have been condensed from those used in the statistical calculations by decreasing the number of intervals and by combining age and sex groups when analysis has shown no significant differences among them. The mean and standard deviation, together with the standard error of each, are given for each subdivision. Median values are added to those tables where the distribution of values is "skew" rather than "normal."

### *Diet records*

The dietary intakes have been calculated almost entirely from the family consumption records obtained by the inventory and purchase method, using the individual records chiefly to check on the equality of distribution of food consumption within the family. While there are shortcomings in such use of family consumption records, the circumstances under which this survey was conducted made them definitely the most accurate dietary information that was obtained. They were collected by the field workers in terms of actual weight, eliminating variability due to degree of cooperation and interest on the part of the housewife. Few of the families had any stores of foodstuffs at either the beginning or the end of the week, and none produced at home a significant amount of the food consumed. The amount of food eaten away from home was negligible.

To express the family intake in terms of individual consumption, the average daily intake of each dietary constituent for the entire family was computed, and that value for calories, carbohydrate, fat, total protein, and animal protein was divided by the number of adult male consumption units in the family. The latter were determined by using the caloric

coefficients of Bigwood ('39). Adjustments were made when indicated by the records of individual consumption; this was most often necessary with respect to milk used in supplementary feedings of nursing infants.

Therefore the values in table 2 represent the average daily intake of the family in terms of an adult male of average size performing moderate work, and the relative adequacy of

TABLE 2

*Daily dietary intake of families in terms of adult male consumption unit.*

| CALORIES | | CARBOHYDRATE | | FAT | | TOTAL PROTEIN | | ANIMAL PROTEIN | |
|---|---|---|---|---|---|---|---|---|---|
| per day | families | gm./day | families | gm./day | families | gm./day | families | gm./day | families |
| Below 750 | 4 | Below 100 | 3 | 5–25 | 30 | Below 25 | 4 | 0– 7.5 | 19 |
| 750– 1250 | 28 | 100– 200 | 43 | 25–50 | 39 | 25– 50 | 29 | 7.5– 20 | 37 |
| 1250– 1750 | 35 | 200– 300 | 34 | 50–75 | 21 | 50– 75 | 36 | 20– 32.5 | 29 |
| 1750– 2250 | 22 | 300– 400 | 15 | 75–100 | 10 | 75– 100 | 23 | 32.5– 45 | 8 |
| 2250– 2750 | 7 | 400– 500 | 4 | Above 100 | 3 | 100– 120 | 2 | 45– 57.5 | 4 |
| Above 2750 | 6 | Above 500 | 3 | | | Above 120 | 2 | 57.5– 70 | 4 |
| | | | | | | | | Above 70 | 2 |
| Total | 102 | | 102 | | 103 | | 103 | | 103 |
| Mean | 1602 ± 61 | 238 ± 10 | | 42 ± 3.0 | | 66 ± 2.7 | | 22 ± 1.7 | |
| Standard deviation | 616 ± 43 | 105 ± 7 | | 30 ± 2.1 | | 27 ± 1.9 | | 17 ± 1.2 | |
| Median | 1543 | 218 | | 37 | | 63 | | 18 | |

the diet for each individual is obtained by comparing the given value with the requirement of such an adult male. This procedure makes it possible to reduce to a common basis the dietary intakes of families differing in age and sex composition, and so differing in caloric and protein requirements. The protein requirement is closely related to that for calories, being dependent on age and weight, and Bigwood's protein

## TABLE 3

*Dietary intake of families per person per day.*

| CALCIUM | | PHOSPHORUS | | IRON | | VITAMIN A | | THIAMINE | | RIBOFLAVIN | | ASCORBIC ACID | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| gm. per head | no of families | gm. per head | no of families | mg per head | no of families | I U. per head | no of families | mg per head | no of families | mg per head | no of families | mg per head | no of families |
| .075– | 17 | .250– | 8 | 3– | 6 | Below 1500 | 17 | .250– | 12 | .100– | 12 | 2– | 11 |
| .150– | 44 | .500– | 17 | 6– | 27 | 1500– | 35 | .500– | 35 | .250– | 46 | 20– | 34 |
| .300– | 26 | .750– | 29 | 9– | 26 | 3000– | 17 | .750– | 30 | .500– | 25 | 40– | 35 |
| .450– | 8 | 1.000– | 20 | 12– | 27 | 4500– | 13 | 1.000– | 15 | .750– | 11 | 60– | 13 |
| .600– | 6 | 1.250– | 19 | 15– | 8 | 6000– | 7 | 1.250– | 8 | 1.000– | 6 | 80– | 4 |
| Above .750 | 2 | 1.500– | 6 | 18– | 5 | 7500– | 7 | Above 1.500 | 2 | Above 1.250 | 3 | 100– | 4 |
|  |  | 1.750–2.000 | 4 | Above 21 | 4 | Above 9000 | 7 |  |  |  |  | Above 120 | 2 |
| **Total families** 103 | | 103 | | 103 | | 103 | | 102 | | 103 | | 103 | |
| **Mean** .301 ± .018 | | 1.021 ± .036 | | 11.4 ± .43 | | 3852 ± 273 | | .820 ± .030 | | .542 ± .029 | | 47.4 ± 2.8 | |
| **Standard deviation** .181 ± .013 | | .366 ± .026 | | 4.3 ± .30 | | 2767 ± 193 | | .304 ± .021 | | .294 ± .020 | | 28.3 ± 2.0 | |
| **Median** .256 | | .983 | | 11.3 | | 2875 | | .794 | | .475 | | 44.2 | |

coefficients correspond almost exactly with his calorie coefficients, so no significant error is introduced by not using a separate system of coefficients for protein.

With respect to mineral and vitamin intake, no satisfactory system of coefficients is available and the requirements show no constant change with age and sex. Accordingly the intake of these constituents (table 3) is expressed as daily intake per person, obtained by dividing the daily average consumption by the number of persons in the family.

The foods of chief importance in determining the caloric intake were bread, which frequently supplied more than one-half of the total calories, olive oil, potatoes, and sometimes rice; occasionally a fresh vegetable was eaten in such enormous quantities that it made a significant contribution to the caloric total. The amount of olive oil consumed virtually determined the fat intake. Animal protein was supplied almost entirely by fish, with milk making an occasional small contribution; meat, eggs, and cheese were practically unknown. Vegetable protein was derived largely from bread, fresh or dried beans, potatoes and garbanzos. The bread was a coarse, dark brown, whole grain product (chiefly wheat) which also contributed significantly to the total mineral intake, especially of iron and phosphorus, and was of paramount importance in determining the thiamine intake. Vitamin A was almost entirely in the form of carotene from fresh vegetables, mainly string beans, lettuce, tomatoes, and chard. These same vegetables plus potatoes and peppers largely determined the ascorbic acid intake; fruit was out of the financial reach of these people. The nicotinic acid content of the diets has not been calculated, as reliable values are available for only a few of the foods consumed. It is probable that the pellagra-preventive value of these diets is limited, and provided chiefly by the fish and green vegetables.

Table 4 indicates the derivation of calories from the three primary food constituents. There is a tendency for carbohydrates to contribute a relatively high proportion of the total energy. There was no definite correlation between the

total calories in the diet and the proportion contributed by any constituent; some of the families in the higher caloric levels got most of the additional calories from carbohydrates, others from a higher intake of olive oil. The proportion of calories derived from protein was relatively constant.

TABLE 4

*Daily dietary intake of families: derivation of calories.*

| PER CENT OF TOTAL CALORIES | NUMBER OF FAMILIES DERIVING STATED PER CENT OF CALORIES FROM: | | |
|---|---|---|---|
| | Carbohydrate | Fat | Protein |
| 5 | — | 4 | — |
| 10 | — | 22 | 11 |
| 15 | — | 19 | 59 |
| 20 | — | 9 | 26 |
| 25 | — | 10 | 5 |
| 30 | 1 | 9 | — |
| 35 | 1 | 10 | 1 |
| 40 | 5 | 11 | — |
| 45 | 9 | 6 | — |
| 50 | 13 | 2 | — |
| 55 | 13 | — | — |
| 60 | 12 | — | — |
| 65 | 9 | — | — |
| 70 | 25 | — | — |
| 75 | 10 | — | — |
| 80 | 3 | — | — |
| 85 | 1 | — | — |
| Total families | 102 | 102 | 102 |
| Mean per cent of total calories | 60.4 ± 1.16 | 23.4 ± 1.24 | 16.4 ± 0.40 |
| Standard deviation | 11.8 ± 0.82 | 12.5 ± 0.87 | 4.0 ± 0.28 |

In considering ·the dietary requirements of this group, certain observations should be mentioned. While no attempt has been made to calculate the energy requirements of each individual, an approximation of the mean and range can be made by using the recorded weights and a rough estimate of the level of activity (table 5). It appears that the figure of

TABLE 5

*Energy requirements based on observed mean height and weight.*[1]

| NUMBER PERSONS AGE 18–49 YEARS | | HEIGHT IN CUBIC METERS | | WEIGHT IN KILOGRAMS | |
|---|---|---|---|---|---|
| | | Mean | Standard deviation | Mean | Standard deviation |
| Males | 70 | 162 ± .7 | 6.2 ± .5 | 54.1 ± .7 | 6.1 ± .5 |
| Females | 111 | 151 ± .6 | 5.9 ± .4 | 46.6 ± .6 | 6.5 ± .4 |

| | HOURS | CALORIES/ KILOGRAMS PER HOUR | CALORIES REQUIRED | | |
|---|---|---|---|---|---|
| | | | Mean weight −2σ | Mean weight | Mean weight +2σ |
| Males | | | | | |
| Sleeping | 8 | 0.93 | 312 | 402 | 493 |
| At rest | 4 | 1.43 | 240 | 309 | 379 |
| Light exercise | 6 | 2.43 | 612 | 789 | 965 |
| Active exercise | 6 | 4.14 | 1043 | 1344 | 1644 |
| Total calories per day | | | 2207 | 2844 | 3481 |
| Females | | | | | |
| Sleeping | 8 | 0.93 | 251 | 347 | 443 |
| At rest | 4 | 1.43 | 193 | 267 | 340 |
| Light exercise | 8 | 2.43 | 655 | 906 | 1157 |
| Active exercise | 4 | 4.14 | 558 | 772 | 985 |
| Total calories per day | | | 1657 | 2292 | 2925 |

[1] Sherman and Langford ( '40).

2,800 calories is justified as the mean energy requirement of the adult males in this study, and should be used for comparison with the values for caloric intake in table 2. This estimated mean requirement also furnishes some justification for the use of Bigwood's coefficients in the calculation of this table.

The data on body weight also furnish a basis for estimating the protein requirement. If the usual figures of ⅔ to 1 gm. of protein for each kilogram of body weight are used, the absolute values for total protein intake are in the range of adequacy for most of the families. However, the relative adequacy of the protein ration is impaired by two other characteristics of the diet: the relatively low proportion of protein from animal sources in the majority of the families,

which opens up the possibility of qualitative protein deficiency, and the almost uniformly low caloric intake, which is known to increase the protein requirement.

This low caloric intake undoubtedly exerts a protective action as far as vitamin and possibly mineral requirements are concerned. The only constituent concerning which this relationship is well enough understood to permit quantitative expression is the association of the thiamine requirement with the intake of nonfat calories (Williams and Spies, '38). The mean ratio of thiamine to nonfat calories for the families studied was $0.70 \pm .018$ with a standard deviation of $0.18 \pm .013$. For none of the families was the ratio 0.3 or less, the level at which clinical beriberi is stated to appear.

In general, the dietary intake of this group may be characterized as far below maintenance in caloric value and calcium content, and relatively inadequate in protein of animal origin. It appears relatively adequate in iron, thiamine, ascorbic acid, and probably phosphorus. Vitamin A and riboflavin levels are low and may well be below minimum maintenance requirements.

The question of how long these people had subsisted on such a regime is of paramount importance if the dietary data are to be correlated with the clinical and laboratory data. It was impossible to obtain reliable individual information on this point. In general, it appeared that these people were eating as good a diet from the qualitative point of view during the survey period as at any time during the past $2\frac{1}{2}$ to 4 years, although the quantity may have been greater and certainly has been less at intervals in the past. It should be borne in mind that these data were collected during the summer months.

### Clinical examination

The following diagnoses of organic disease were made on the basis of the history and physical examination:

NUMBER OF
PERSONS

| | |
|---|---|
| Definite tuberculosis | 13 |
| Suspected tuberculosis | 17 |
| Syphilis | 12 |
| Rheumatic heart disease | 7 |
| Gall-bladder disease | 6 |
| Hypertension | 4 |
| Progressive muscular dystrophy | 3 (all brothers) |
| Malaria | 2 |
| Bacterial endocarditis, hepatic cirrhosis, and perinephric abscess | 1 each |

Among the women in the reproductive age period, two were pregnant and twenty-nine were lactating. The data on persons with active infections or metabolic disorders are not included in the tables of clinical and laboratory findings; with respect to other conditions, findings which may be due to the concomitant disease or state are excluded.

The incidence of classical deficiency disease was slight: there were two cases of definite nutritional edema, one subject with glossitis, and a chronic pellagrous dermatitis; among twenty children under 2 years of age on whom x-ray films were obtained two cases of active rickets were diagnosed. No cases of xerophthalmia, scurvy, beriberi, or specific cheilosis were seen.

The incidence of symptoms and signs which have been attributed to general undernutrition, or suggested as early manifestations of specific deficiencies, is indicated in table 6. Since many of these are not specific or their significance is controversial, the actual complaints and findings are listed. It should be borne in mind that these are subjects in whom organic disease has been excluded, in so far as this is possible, by means of clinical examination.

The most frequent item was a history of weight loss, supported by the finding of a large number with evidence thereof, chiefly in the form of scant subcutaneous tissue. A number of the children were judged to be poorly developed, and over one-third were noted to have flaring rib margins and flaccid abdominal musculature. More specific deformities of healed

TABLE 8

*Incidence of symptoms and physical findings attributable to nutritional deficiency.*

| | UNDER 13 YEARS | | | | | OVER 13 YEARS | | | |
| | AGE IN MONTHS 2-24 | 2-5 | 6-12 | Total | | Males | Females | Total | |
| | | | | Number | Per cent | | | Number | Per cent |
|---|---|---|---|---|---|---|---|---|---|
| Total persons | 39 | 63 | 155 | 257 | 100 | 110 | 160 | 270 | 100 |
| **Symptoms** | | | | | | | | | |
| Weight loss | 18 | 37 | 125 | 180 | 70 | 83 | 131 | 214 | 79 |
| Weakness and/or ease of fatigue | 4 | 22 | 102 | 128 | 50 | 80 | 133 | 213 | 79 |
| Irritability | 3 | 6 | 20 | 29 | 11 | 10 | 28 | 38 | 14 |
| Psychic changes | — | 2 | 4 | 6 | 2 | 13 | 32 | 45 | 17 |
| Unexplained secondary amenorrhea | — | — | — | — | — | — | 11 | 11 | 7[1] |
| Night blindness | — | — | — | — | — | 4 | 9 | 13 | 5 |
| Paresthesias and pain in legs | — | 11 | 68 | 79 | 31 | 62 | 113 | 175 | 65 |
| Photophobia | — | 2 | 15 | 17 | 7 | 16 | 30 | 46 | 17 |
| Sore mouth | — | 1 | 9 | 10 | 4 | 6 | 12 | 18 | 7 |
| Gastrointestinal symptoms | — | — | 11 | 11 | 4 | 14 | 25 | 39 | 14 |
| Bleeding gums | — | 1 | 10 | 11 | 4 | 8 | 12 | 20 | 7 |
| Edema | — | — | 2 | 2 | 1 | 9 | 24 | 33 | 12 |
| **Physical findings** | | | | | | | | | |
| Scant subcutaneous fat | 14 | 19 | 98 | 131 | 51 | 47 | 69 | 116 | 43 |
| Poor physical development | 12 | 8 | 28 | 48 | 19 | 6 | 4 | 10 | 4 |
| Flaring costal arch, pot belly | 9 | 39 | 43 | 91 | 35 | — | — | — | — |
| Hyperkeratosis pilaris | — | 6 | 40 | 46 | 18 | 8 | 18 | 26 | 10 |
| Calf muscle tenderness | — | 2 | 25 | 27 | 10 | 32 | 49 | 81 | 30 |
| Atrophy lingual papillae | — | 3 | 17 | 20 | 8 | 10 | 31 | 41 | 15 |
| Inflammation oral mucosa | — | — | — | — | — | 12 | 13 | 25 | 9 |
| Minor skin lesions | 1 | 16 | 78 | 95 | 37 | 39 | 69 | 108 | 40 |
| Bleeding gums | — | — | 5 | 5 | 2 | 1 | 4 | 5 | 2 |
| Edema, minimal | — | — | — | — | — | 1 | 3 | 4 | 1 |
| Edema, frank | — | — | — | — | — | 1 | 1 | 2 | 1 |

[1] Females over 13 years of age.

rickets, such as rachitic rosary and cranial bossae, were rarely encountered. More than three-fourths of the subjects over 5 years of age complained of weakness and ease of fatigue which they claimed was a definite change from their previous status. A significant number complained of being more irritable than previously. The psychic disturbances complained of were usually apathy in the children and loss of memory in the adults. Of interest is the occurrence of secondary amenorrhea in eleven women between the ages of 18 and 36, with no other apparent physiologic or pathologic explanation.

With respect to possible vitamin A deficiency, thirteen persons complained of night blindness and seventy-two presented skin lesions with horny spines in the hair follicles. No tests of dark adaptation were made. The skin lesions were usually seen on the lateral aspect of the arms and thighs, although in a few instances they were confined to the abdomen. The condition showed a definite tendency to occur in several members of the same family.

A surprisingly large number of subjects complained of paresthesias in the extremities and pain or aching in the muscles; this was associated with the finding of calf muscle tenderness in many of these persons. In the United States, these symptoms and findings would usually be attributed to a thiamine deficiency, but there are two reasons for questioning such an interpretation in the present study. The first is the relative adequacy of the thiamine intake indicated by the dietary records. The second is concerned with observations made in Madrid during the civil war (Grande Covian and Jiménez Garcia, '40, I and II; Grande and Peraita, '41). During the food restriction incident to the siege of Madrid, over 700 cases of a severe paresthetic syndrome were studied carefully, none of which presented or developed characteristic motor or reflex changes of peripheral neuritis. The peak of occurrence of this condition was closely related to an epidemic of pellagra. The condition did not respond to thiamine, but was benefited by the administration of yeast. These experiences suggest that some other factor in the vita-

min B complex is involved. The dietary records lend support
to the possibility that calcium deficiency may have been a
factor in the findings in the present study.

Symptoms which have been suggested as early manifesta-
tions of a vitamin B complex deficiency of a pellagrous nature
occurred in relatively few of the subjects. Little reliance
could be placed on gastrointestinal complaints, because of a
smoldering epidemic of infectious gastroenteritis in this area
throughout the summer. Atrophy of the lingual papillae and
mild inflammation of the oral mucosa were observed chiefly in
adults. A large number of subjects presented minor skin
lesions, such as thickening over the pressure points, loss of
elasticity, scaling, etc. The circumstances under which these
subjects were living (much exposure to sun and dirt, the virtual
absence of soap, a high incidence of scabies and louse in-
festation) make it impossible to rely on such observations as
manifestations of a deficiency state.

The serum ascorbic acid levels gave no grounds for at-
tributing the infrequent complaint or finding of bleeding gums
to a vitamin C deficiency.

Although thirty-five subjects gave a history of recent edema,
only four cases of minimal and two cases of frank edema were
encountered on examination; none of these had albuminuria
or heart disease. The two cases of frank edema were definitely
of nutritional origin, occurring in the father and mother of
a family whose dietary record indicated a daily average
intake per adult male unit of 750 calories and 33 gm. of
protein, none of animal origin. The serum protein values
per 100 cc. were 5.9 gm. total protein for the man, with 3.5
gm. of albumin, and 6.9 gm. total for the woman, with 3.8 gm.
of albumin.

The value of the recorded body weights and measurements
is impaired by the absence of such data on normal persons of
Spanish stock. The weights of the subjects free from organic
disease are compared with American standards for sex, height,
and age in table 7. Of the 521 individuals, 332 were more than
7.5% underweight, and 213 more than 12.5% underweight.

TABLE 7

*Body weight. Per cent variation from American standards [1] for height, age, and sex.*

|  | 0–24 MONTHS | 2–5 YEARS | 6–12 YEARS | 13–19 YEARS | 20–49 YEARS | OVER 49 YEARS | TOTAL |
|---|---|---|---|---|---|---|---|
| *per cent* |  |  |  |  |  |  |  |
| Above +12.5 | — | 1 | — | 3 | 1 | — | 5 |
| +12.5 to  +7.5 | 4 | – | 2 | 4 | — | - - | 10 |
| +7.5 ''  +2.5 | 4 | 3 | 7 | 2 | 4 | — | 20 |
| +2.5 ''  —2.5 | 3 | 12 | 16 | 12 | 15 | 1 | 59 |
| —2.5 ''  —7.5 | 7 | 16 | 37 | 16 | 19 | — | 95 |
| —7.5 ''—12.5 | 8 | 15 | 44 | 16 | 32 | 4 | 119 |
| —12.5 ''—17.5 | 4 | 12 | 37 | 11 | 37 | 2 | 103 |
| —17.5 ''—22.5 | 3 | 2 | 10 | 8 | 24 | 6 | 53 |
| —22.5 ''—27.5 | 2 | - - | 3 | 5 | 14 | 6 | 30 |
| —27.5 ''—32.5 | 3 | - - | — | — | 12 | 4 | 19 |
| Below —32.5 | — | — | 1 | — | 5 | 2 | 8 |
| Total | 38 | 61 | 157 | 77 | 163 | 25 | 521 |
| Mean | —8.3 ±1.9 | —6.8 ±.9 | —9.1 ±.6 | —7.6 ±1.1 | —14.1 ±.8 | —21.4 ±1.8 | —10.7 ±.4 |
| Standard deviation | 11.5 ±1.4 | 7.1 ±.6 | 7.1 ± 4 | 9 9 ±.8 | 9.6 ±.5 | 9.1 +1.3 | 9.6 ± 3 |

[1] Standards obtained from following tables:

> Birth to 5 years..     R. M. Woodbury
> 6 to 18 years .     B. T. Baldwin and T. D. Wood
> Above 18 years     T. D. Wood

Additional evidence of general quantitative undernutrition (Benedict, '19) is seen in the tendency of the blood pressures of these subjects to be low (table 8).

The findings concerning capillary invasion of the cornea are recorded in an arbitrary descriptive classification in table 9. A number of subjects had evident conjunctival disease, usually trachoma, which may have accounted for the invasion seen. Sixteen per cent of the males and 26% of the females had findings which may be attributed to active ariboflavinosis (Sydenstricker, Sebrell, Cleckley and Kruse, '40) and an additional 12 and 10%, respectively, had findings typical of previous or disappearing deficiency. We feel that there is some uncertainty regarding the significance of invasion limited to the nasal quadrant. A higher incidence of

TABLE 8

*Blood pressure.*

| SYSTOLIC | 13–20 YEARS | | 21–39 YEARS | | 40–78 YEARS | |
|---|---|---|---|---|---|---|
| | Male | Female | Male | Female | Male | Female |
| (mm Hg) | | | | | | |
| 75– 84 | | | | | | |
| 85– 94 | 4 | 3 | 1 | 1 | 4 | 2 |
| 95–104 | 12 | 12 | 6 | 8 | 5 | 7 |
| 105–114 | 7 | 14 | 9 | 15 | 4 | 6 |
| 115–124 | 2 | 1 | 4 | 10 | 10 | 8 |
| 125–134 | — | 1 | 5 | 8 | 5 | 3 |
| 135–144 | — | 1 | — | 1 | 1 | 3 |
| 145–154 | — | — | — | — | 1 | 2 |
| Over 154 | — | — | — | — | — | 5 |
| Total | 26 | 32 | 25 | 43 | 30 | 36 |
| Mean | 102 ± 1.8 | 106 ± 1.8 | 112 ± 2.3 | 114 ± 1.7 | 115 ± 2.8 | 124 ± 4 0 |
| St. dev. | 9 4 ± 1.3 | 10 4 ± 1.3 | 11.7 ± 1.7 | 11.4 ± 1.2 | 15.5 ± 2 0 | 24.2 ± 2 9 |
| DIASTOLIC (mm Hg) | | | | | | |
| 45– 54 | 5 | 2 | 1 | 1 | 2 | 2 |
| 55– 64 | 14 | 11 | 6 | 6 | 7 | 2 |
| 65– 74 | 5 | 14 | 10 | 13 | 9 | 12 |
| 75– 84 | 2 | 5 | 7 | 19 | 12 | 11 |
| 85– 94 | — | — | 1 | 4 | — | 8 |
| 95–105 | — | — | — | — | — | 1 |
| | 26 | 32 | 25 | 43 | 30 | 36 |
| Mean | 62 ± 1 6 | 67 ± 1.5 | 70 ± 1.9 | 74 ± 1.4 | 70 ± 1.8 | 77 ± 1.9 |
| St. dev. | 8 3 ± 1.2 | 8.2 ± 1.0 | 9.4 ± 1.3 | 9.3 ± 1.0 | 9.6 ± 1.2 | 11.5 ± 1.4 |

capillary invasion is apparent in females of all ages; the incidence was no higher among the lactating women than among nonlactating women in the same age group, although the most severe cases were seen in the former group. While the number of persons over 50 years of age is small, there is a suggestion of lower incidence in this age group. Sydenstricker and collaborators have remarked on the possible effect of arcus senilis in preventing capillary invasion. As Youmans ('41) found in Marseille, the symptom of photophobia was of little help in predicting the slit lamp findings.

TABLE 9

*Capillary invasion of the cornea.*

| Sex | Males | | | | | | Females | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Total | | | | | | Total | |
| Age in years | 6–12 | 13–20 | 21–49 | Over 49 | Number | Per cent | 6–12 | 13–20 | 21–49 | Over 49 | Number | Per cent |
| *Invasion* | | | | | | | | | | | | |
| None | 39 | 23 | 33 | 7 | 102 | 57 | 21 | 15 | 38 | 12 | 86 | 38 |
| Nasal quadrant only | 7 | 2 | 9 | 1 | 19 | 11 | 16 | 7 | 16 | 2 | 41 | 18 |
| More than nasal quadrant | | | | | | | | | | | | |
| Less than 25% | 9 | 4 | 8 | – | 21 | 12 | 6 | 4 | 11 | 1 | 22 | 10 |
| More than 25% | 7 | 8 | 12 | 1 | 28 | 16 | 16 | 14 | 27 | 2 | 59 | 26 |
| Other eye disease | 4 | 2 | 3 | – | 5 | 5 | 3 | 3 | 11 | 3 | 20 | 9 |
| Total persons examined | 66 | 39 | 65 | 9 | 179 | 100 | 62 | 43 | 103 | 20 | 228 | 100 |

| Riboflavin intake | Males | | Females | |
|---|---|---|---|---|
| | Below 0.475 mg. | Above 0.475 mg. | Below 0.475 mg. | Above 0.475 mg. |
| *Invasion* | | | | |
| None | 37 | 50 | 22 | 48 |
| Nasal quadrant only | 7 | 10 | 16 | 20 |
| More than nasal quadrant | | | | |
| Less than 25% | 9 | 7 | 14 | 8 |
| More than 25% | 12 | 14 | 38 | 12 |
| Other eye disease | 5 | 7 | 8 | 10 |
| Total persons under 50 years of age examined | 70 | 88 | 98 | 98 |

To investigate crudely the relationship between riboflavin intake and corneal invasion, the subjects under 50 years of age have been grouped according to riboflavin intake shown in the family diet record, the individuals being classified as they fell above or below the median value. The results are shown in table 9. The correlation between low levels of riboflavin intake and the incidence of corneal invasion seems quite definite among the females, but is not apparent among the males.

X-rays revealed active rickets in two of the twenty children between the ages of 3 to 24 months on whom the reports of the films are available. In addition, the x-ray showed definite evidence of incompletely healed rickets in one child 3 years old. No active or healed scorbutic lesions were seen by x-ray. The serum phosphatase level was definitely abnormal in only one child among the fifty-eight under the age of 6 years on whom this determination was made. This child had active rickets by x-ray and a serum phosphatase activity of 27 Bodansky units per 100 cc. of serum. No determination was made on the other child with active rickets.

The growth and developmental status of the children, as evidenced by weight, body measurements, and x-ray studies, are reported in a separate communication (Robinson, Janney and Grande Covian, '42). In general, the children included in this survey are from 1 to 3 or more years behind American children of the same age with respect to weight, height, and skeletal maturation, and there is x-ray evidence of repeated disturbances of growth of the long bones.

### Laboratory determinations

The hematologic determinations and the measurements derived from them are presented in tables 10 to 12. Additional calculations of the values for the women who were lactating compared to those of nonlactating controls of the same age indicate that this condition was not a significant factor affecting the values obtained on the women during the reproductive age period.

Table 10 shows that 16% of the males and 18% of the females had hemoglobin values of less than 12 gm. per 100 cc. This does not suggest a high incidence of anemia in the population. However, in table 11 it is seen that 31% of the males and 33% of the females had red blood cell counts of less than 4,125,000, and that the values for males fail to show the normal tendency to be higher than those for females. Table 12 shows

TABLE 10

*Hemoglobin.*

| GRAMS PER 100 CC. | MALES | | | | | FEMALES | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2–24 months | 2–12 years | 13–20 years | 21–60 years | Total | 2–24 months | 2–12 years | 13–20 years | 21–49 years | Over 49 years | Total |
| Below 10.5 | — | — | — | — | — | 1 | — | — | 1 | — | 2 |
| 10.5–12.0 | 4 | 21 | 4 | 6 | 35 | 6 | 26 | — | 13 | 2 | 47 |
| 12.0–13.5 | 6 | 54 | 17 | 14 | 91 | 8 | 56 | 17 | 44 | 7 | 132 |
| 13.5–15.0 | 1 | 20 | 16 | 30 | 67 | 1 | 20 | 20 | 40 | 2 | 83 |
| 15.0–16.5 | — | 4 | 2 | 14 | 20 | — | 3 | 3 | 4 | 4 | 14 |
| Above 16.5 | — | 1 | — | 3 | 4 | — | — | — | — | — | — |
| Total | 11 | 100 | 39 | 67 | 217 | 16 | 105 | 40 | 102 | 15 | 278 |
| Mean | 12.5 ±.26 | 12.8 ±.11 | 13.4 ±.18 | 14.1 ±.17 | 13.3 ±.09 | 12.0 ±.28 | 12.7 ±.10 | 13.7 ±.14 | 13.2 ±.11 | 13.7 ±.34 | 13.5 ±.07 |
| Standard deviation | .9 ±.18 | 1.1 ±.08 | 1.1 ±.13 | 1.4 ±.12 | 1 4 ±.07 | 1.1 ±.19 | 1.0 ±.07 | 0.9 ±.10 | 1.1 ±.08 | 1.3 ±.24 | 1.1 ±.05 |

TABLE 11

*Red blood cell counts.*

| ERYTHROCYTE COUNT IN MILLIONS PER MM³. | MALES | | | | | FEMALES | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2–24 months | 2–12 years | 13–20 years | Over 20 years | Total | 2–24 months | 2–12 years | 13–20 years | Over 20 years | Total |
| Below 3.62 | 1 | — | 3 | 6 | 10 | 1 | 1 | 1 | 6 | 9 |
| 3.63–4.12 | 1 | 35 | 8 | 14 | 58 | 5 | 34 | 11 | 32 | 82 |
| 4.13–4.62 | 5 | 49 | 22 | 28 | 104 | 5 | 51 | 18 | 55 | 129 |
| 4.63–5.12 | 4 | 14 | 6 | 15 | 39 | 5 | 16 | 9 | 24 | 54 |
| 5.13–5.50 | — | 2 | — | 3 | 5 | — | 2 | 1 | — | 3 |
| Total | 11 | 100 | 39 | 66 | 216 | 16 | 104 | 40 | 117 | 277 |
| Mean | 4.45 ±.15 | 4.29 ±.04 | 4.28 ±.06 | 4.34 ±.06 | 4.31 ±.03 | 4.31 ±.10 | 4.29 ±.04 | 4.37 ±.06 | 4.29 ±.04 | 4.30 ±.02 |
| Standard deviation | 0.48 ±.10 | 0.38 ±.03 | 0.39 ±.04 | 0.53 ±.05 | 0.43 ±.02 | 0.38 ±.07 | 0.38 ±.03 | 0.39 ±.04 | 0.38 ±.03 | 0.38 ±.02 |

## TABLE 12
*Hematologic measurements.*

| Sex | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|
| Age in years | 0–12 | 13–20 | Over 20 | Total | 0–12 | 13–20 | Over 20 | Total |
| **Mean corpuscular volume** | | | | | | | | |
| *cubic micra* | | | | | | | | |
| Below 74 | 1 | — | — | 1 | — | — | — | — |
| 74– 79 | 6 | — | — | 6 | 7 | — | 4 | 11 |
| 80– 85 | 27 | 5 | 3 | 35 | 33 | 2 | 10 | 45 |
| 86– 88 | 16 | 11 | 4 | 31 | 19 | 10 | 22 | 51 |
| 89– 94 | 41 | 10 | 22 | 73 | 38 | 14 | 40 | 92 |
| 95–100 | 13 | 7 | 18 | 38 | 16 | 6 | 25 | 47 |
| 101–106 | 6 | 4 | 10 | 20 | 4 | 4 | 12 | 20 |
| Above 106 | 1 | 2 | 9 | 12 | 1 | 4 | 2 | 7 |
| Total | 111 | 39 | 66 | 216 | 118 | 40 | 115 | 273 |
| Mean | 89±.7 | 92±1.1 | 97±1.0 | 92±.5 | 89±.6 | 94±1.3 | 92±.7 | 91±.4 |
| Standard deviation | 7±.5 | 7± .8 | 8± .7 | 8±.4 | 6±.4 | 8± .9 | 7±.5 | 7±.3 |
| **Mean corpuscular hemoglobin** | | | | | | | | |
| *micro-micrograms* | | | | | | | | |
| Below 24 | — | — | — | — | 1 | — | 1 | 2 |
| 24–26 | 6 | — | — | 6 | 15 | — | 5 | 20 |
| 27–29 | 48 | 9 | 6 | 63 | 38 | 9 | 20 | 67 |
| 30–32 | 39 | 20 | 31 | 90 | 54 | 20 | 59 | 133 |
| 33–35 | 11 | 7 | 14 | 32 | 9 | 7 | 23 | 39 |
| 36–38 | 4 | 3 | 11 | 18 | 1 | 4 | 5 | 10 |
| Above 38 | 1 | — | 2 | 3 | 1 | — | — | 1 |
| Total | 109 | 39 | 64 | 212 | 119 | 40 | 113 | 272 |
| Mean | 29.9±.26 | 31.2±.45 | 32.7±.47 | 31.0±.21 | 29.6±.23 | 31.3±.39 | 30.9±.25 | 30.4±.16 |
| Standard deviation | 2.8±.19 | 2.8±.32 | 3.8±.33 | 3.0±.15 | 2.6±.17 | 2.5±.28 | 2.7±.18 | 2.6±.11 |
| **Mean corpuscular hemoglobin concentration** | | | | | | | | |
| *per cent* | | | | | | | | |
| Below 30 | 1 | — | 2 | 3 | 2 | 1 | 2 | 5 |
| 30–31 | 5 | 2 | 3 | 10 | 9 | 3 | 5 | 17 |
| 32–33 | 50 | 14 | 24 | 88 | 58 | 14 | 52 | 124 |
| 34–35 | 41 | 19 | 32 | 92 | 43 | 22 | 45 | 110 |
| 36–37 | 9 | 4 | 3 | 16 | 5 | — | 7 | 12 |
| 38–39 | 3 | — | 1 | 4 | — | — | — | — |
| Above 39 | 1 | — | 1 | 2 | — | — | — | — |
| Total | 110 | 39 | 66 | 215 | 117 | 40 | 111 | 268 |
| Mean | 33.7±.17 | 93.8±.21 | 33.8±.23 | 33.7±.12 | 33.2±.14 | 33.4±.22 | 33.4±.14 | 53.3±.09 |
| Standard deviation | 1.8±.12 | 1.3±.15 | 1.8±.16 | 1.8±.08 | 1.5±.10 | 1.4±.16 | 1.5±.10 | 1.5±.06 |

that, whereas only 3% of males and 4% of females had values for mean corpuscular volume of less than 80 cubic micra, 32% of the males and 27% of females had values above 94 cubic micra. Corresponding percentages of abnormal values (Wintrobe, '29) for mean corpuscular hemoglobin are 3% of males and 8% of females with values below 27 micromicrograms, but 25% of males and 18% of females with values above 32 micromicrograms. Most of the low values for these determinations occurred among the children examined. Most of the values for mean corpuscular hemoglobin concentration fell between 32 and 37%.

TABLE 13

*Serum proteins.*

| TOTAL PROTEIN | MALES | FEMALES | TOTAL | ALBUMIN | MALES | FEMALES | TOTAL |
|---|---|---|---|---|---|---|---|
| *gm /100 cc serum* | | | | *gm /100 cc serum* | | | |
| Below 5.50 | 1 | 2 | 3 | Below 3.50 | 2 | 1 | 3 |
| 5.50–6.00 | 11 | 1 | 12 | 3.50–4.00 | 20 | 11 | 31 |
| 6.00–6.50 | 43 | 43 | 86 | 4.00–4.50 | 45 | 64 | 109 |
| 6.50–7.00 | 54 | 73 | 127 | 4.50–5.00 | 75 | 100 | 175 |
| 7.00–7.50 | 66 | 95 | 161 | 5.00–5 50 | 41 | 60 | 101 |
| 7.50–8 00 | 22 | 33 | 55 | Above 5.50 | 13 | 28 | 41 |
| Above 8.00 | 6 | 20 | 26 | | | | |
| Total | 203 · | 267 | 470 | Total | 196 | 264 | 460 |
| Mean | 6.90±.04 | 7.07±.04 | 7.00±.03 | Mean | 4 69±.04 | 4.81±.03 | 4.76± 03 |
| Standard deviation | .60±.03 | .60±.03 | 60±.02 | Standard deviation | .55±.03 | .54±.02 | .55±.02 |

The chief hematologic abnormality is a macrocytic hyperchromic anemia. This is definitely not an iron deficiency manifestation. The association of such macrocytic anemias with diets inadequate in protein, especially animal protein, has been noted (Bethell, '36).

The levels of serum albumin are probably the most significant values presented in table 13. Eleven per cent of the males and 5% of the females had values for serum albumin below 4 gm. per 100 cc. Of the thirty-four persons with serum albumin

below this level, only five had a total protein content of less than 6 gm. per 100 cc. of serum, indicating the necessity of determining the serum protein fractions in testing for hypoproteinemia. The values for serum globulin are not presented in detail; the mean was 2.28 ± .03 gm. per 100 cc., standard deviation .67 ± .02, with no significant age or sex differences.

TABLE 14

*Serum vitamin A.*

| INTER-NATIONAL UNITS PER 100 CC SERUM | MALES | | | | FEMALES | | | |
|---|---|---|---|---|---|---|---|---|
| | 0–5 years | 6–12 years | Over 12 years | Total | 0–5 years | 6–12 years | Over 12 years | Total |
| Below 35 | 4 | 9 | 4 | 17 | 4 | 8 | 5 | 17 |
| 35– 54 | 11 | 13 | 9 | 33 | 9 | 21 | 13 | 43 |
| 55– 74 | 4 | 12 | 17 | 33 | 7 | 17 | 48 | 72 |
| 75– 94 | 2 | 9 | 16 | 27 | 3 | 7 | 22 | 32 |
| 95–114 | 1 | 5 | 17 | 23 | 2 | 5 | 21 | 28 |
| 115–134 | — | 2 | 7 | 9 | 1 | 3 | 4 | 8 |
| Above 134 | 1 | 2 | 6 | 9 | — | — | 4 | 4 |
| Total | 23 | 52 | 76 | 151 | 26 | 61 | 117 | 204 |
| Mean | 57 ±6 2 | 67 ±4.5 | 87 ±4 1 | 75 ±2.9 | 58 ±5 2 | 62 ±3.4 | 77 ±2.5 | 71 ±2 0 |
| Standard deviation | 30 ±4.4 | 32 ±3.2 | 36 ±2.9 | 35 ±2.0 | 27 ±3.7 | 26 ±2.4 | 28 ±1.8 | 28 ±1.4 |
| Median | 51 | 68 | 83 | 71 | 56 | 59 | 71 | 68 |

Generally accepted standards for a satisfactory level of vitamin A in the serum are at present not definitely established. Most persons eating a well-balanced diet have values in the neighborhood of 100 I.U. per 100 cc. (Kimble, '39). In the present study (table 14) about one-third of the subjects had values below 55 I.U. per 100 cc. The values tend to be lower in children, and there is a probably significant sex difference among the adults, the values for males tending to be higher. The serum carotene levels show a wide range (table 15); no consistent relationship between the carotene and vitamin A levels in the serum of the same individual was noted. Both values tend to be low in the youngest age group.

TABLE 15

*Serum carotene.*

| CONCENTRATION IN MICROGRAMS PER 100 CC. SERUM | 0–5 YEARS | 6–12 YEARS | 13–70 YEARS | TOTAL ALL AGES |
|---|---|---|---|---|
| Below 50 | 9 | 7 | 5 | 21 |
| 50–100 | 16 | 18 | 23 | 57 |
| 100–150 | 13 | 29 | 45 | 87 |
| 150–200 | 4 | 28 | 45 | 77 |
| 200–250 | 4 | 18 | 35 | 57 |
| 250–300 | 2 | 10 | 21 | 33 |
| 300–350 | — | 4 | 12 | 16 |
| 350–400 | — | 2 | 5 | 7 |
| Above 400 | 1 | 4 | 5 | 10 |
| Total | 49 | 120 | 196 | 365 |
| Mean | $116 \pm 12$ | $174 \pm 8$ | $191 \pm 6$ | $175 \pm 5$ |
| Standard deviation | $84 \pm 8$ | $93 \pm 6$ | $87 \pm 4$ | $92 \pm 3$ |
| Median | 98 | 164 | 182 | 164 |

TABLE 16

*Serum ascorbic acid.*

| MG./100 CC. SERUM | ALL SUBJECTS |
|---|---|
| 0.1–0.3 | 1 |
| 0.3–0.5 | 9 |
| 0.5–0.7 | 60 |
| 0.7–0.9 | 140 |
| 0.9–1.1 | 107 |
| 1.1–1.3 | 74 |
| 1.3–1.5 | 33 |
| 1.5–1.7 | 6 |
| Total | 430 |
| Mean | $0.94 \pm 0.12$ |
| Standard deviation | $.24 \pm .008$ |

The ascorbic acid content of the sera (table 16) indicates that nearly all the subjects were receiving an adequate intake of this vitamin.

## DISCUSSION

The clinical and laboratory findings agree fairly well with what might be anticipated from the dietary records. It should be appreciated that the techniques used or available are far from equal in sensitivity in detecting early states of specific deficiency. For example, it is possible to detect vitamin C subsaturation long before clinical scurvy is imminent, but a prolonged and severe restriction of dietary protein is apparently required before the level of serum proteins will fall. The absence of reliable clinical or laboratory criteria of early nicotinic acid deficiency is keenly felt in a study of this sort. These factors, in addition to the protective effect of the low caloric content of the diet, undoubtedly affect the apparent incidence of specific qualitative deficiencies.

These observations, obviously of a localized and seasonal nature, should not be regarded as characteristic of the entire country. Observations by one of us (J.H.J.) during the fall of 1940 indicated that the food supply and state of nutrition varied a great deal from one section of the country to another. The section of nutrition in Madrid is continuing to function in collaboration with the public health authorities, and it is intended to conduct additional surveys during the coming year, as well as evaluate more adequately some of the clinical findings in the original survey by observing response to specific therapy.

## SUMMARY

A study of the nutritional status of 561 persons of a low economic level was carried out in an industrial suburb of Madrid during the summer of 1941 by the combined methods of family food consumption records, individual clinical examination, and laboratory determinations.

The dietary records indicated an average intake of calories and calcium far below maintenance levels, and a protein intake of questionable adequacy. The levels of iron, phosphorus, thiamine, and ascorbic acid appeared generally adequate for maintenance; those for vitamin A and riboflavin at, or below, the border line.

Clinical examination revealed almost universal evidence of quantitative (caloric) underfeeding. Classical qualitative deficiency disease was rare: two cases of nutritional edema, one of pellagra (chronic), three of rickets. Search for early manifestations of such qualitative deficiencies disclosed a significant number of persons with skin lesions attributable to vitamin A deficiency, and a fairly high incidence, especially among the females, of capillary invasion of the cornea which is probably due to ariboflavinosis. In addition, many subjects had signs and symptoms of a mild neural or neuromuscular disturbance of undetermined origin.

Laboratory studies showed that about one-third of the subjects had a mild macrocytic hyperchromic anemia. The incidence of hypoproteinemia was 11% among males and 5% among females. Serum vitamin A levels suggest that at least one-third of the subjects may have been inadequately fed in this respect at the time of the survey. Serum ascorbic acid values indicate that nearly all had adequate body stores of this vitamin.

### LITERATURE CITED

BENEDICT, F. G., AND COLLAB. 1919 Human vitality and efficiency under prolonged restricted diet. Carnegie Institution Publ. No. 280, Washington.

BETHELL, F. H. 1936 The blood changes in normal pregnancy and their relation to the iron and protein supplied by the diet. J. Am. Med. Assoc., vol. 107, p. 564.

BIGWOOD, E.-J. 1939 Guiding principles of studies on the nutrition of populations. League of Nations Technical Commission on Nutrition, Geneva.

BODANSKY, A. 1933 Phosphatase studies; determination of serum phosphatase. J. Biol. Chem., vol. 101, p. 93.

BOOHER, L. E., AND E. R. HARTZLER 1939 The vitamin $B_1$ content of foods in terms of crystalline thiamin. U. S. Dept. of Agriculture, Technical Bulletin No. 707, Washington.

DROESE, W., AND H. BRAMSELL 1941 Vitamin-Tabellen der Gebrauchlichstem Nahrungsmittel. J. A. Barth, Leipzig.

EVELYN, K. A. 1936 A stabilized photoelectric colorimeter with light filters. J. Biol. Chem., vol. 115, p. 63.

FARMER, C. J., AND A. F. ABT 1935 Ascorbic acid content of blood. Proc. Soc. Exp. Biol. and Med., vol. 32, p. 1625.

FISKE, C. H., AND Y. SUBBAROW 1925 Colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

FIXSEN, M. A. B., AND M. H. ROSCOE 1940 Tables of the vitamin content of human and animal foods. II. Nutrition Abstracts and Reviews, vol. 9, p. 795.

GRANDE (COVIAN), F. 1939 La alimentación en Madrid durante la guerra. Publicaciones de la Revista de Sanidad e Higiene Pública, December.

GRANDE (COVIAN), F., AND F. JIMÉNEZ GARCIA 1940 Sobre los trastornos carenciales observados en Madrid durante la guerra. I. Los cuadros clínicos presentados con más frecuencia y su clasificación. II. La presentación en el tiempo de los distintos cuadros carenciales. Revista Clínica Española, vol. 1.

GRANDE, F., AND M. PERAITA 1941 Vitaminas y sistema nervioso. Revista Clínica Española, vol. 2, p. 209.

KIMBLE, M. S. 1939 The photocolorimetric determination of vitamin A and carotene in human plasma. J. Lab. and Clin. Med., vol. 24, p. 1055.

MAY, C. D., K. D. BLACKFAN, J. F. MCCREARY AND F. H. ALLEN 1940 Clinical studies of vitamin A in infants and children. Am. J. Dis. Children, vol. 59, p. 1167.

PUYAL, J., AND SRTA. TORRES 1932 Anales de la Casa de Salud, Valdecilla, Inst'o Medico de Postgraduados, Santander, pp. 191, 197, 213, 278.

RANDOIN, G. L. 1939 Problèmes Actuelles d'Alimentation Herrman, Paris.

ROBINSON, H. W., AND C. G. HOGDEN 1940 Biuret reaction in determination of serum proteins; study of conditions necessary for production of stable color which bears quantitative relationship to protein concentration. J. Biol. Chem., vol. 135, p. 707.

ROBINSON, W. D., J. H. JANNEY AND F. GRANDE COVIAN 1942 Studies of the physical characteristics of selected children in Madrid, Spain, in 1941. J. Pediatrics, vol. 20, p. 723.

SCHALL, H. 1941 Nahrungsmittel-tabelle zur Aufstellung und Berechnung von Diätverordnungen für Krankenhaus Sanat. und Praxis. J. A. Barth, Leipzig.

SHERMAN, H. C. 1937 Chemistry of food and nutrition. 5th ed. Macmillan, New York.

SHERMAN, H. C., AND C. S. LANGFORD 1940 Essentials of nutrition. Macmillan, New York, p. 77.

SYDENSTRICKER, V. P., W. H. SEBRELL, H. M. CLECKLEY AND H. D. KRUSE 1940 The ocular manifestations of ariboflavinosis. J. Am. Med. Assoc., vol. 114, p. 2437.

VAZQUEZ Y SANCHEZ, J. 1932 Composición química de los alimentos españoles. Gaceta Médica Española, Madrid, vol. 6, p. 62.

WILLIAMS, R. R., AND T. D. SPIES 1938 Vitamin $B_1$ (thiamin) and its use in medicine.' Macmillan, New York.

WINTROBE, M. M. 1929 Volume and hemoglobin content of red blood corpuscle: simple method of calculation, normal findings, and value of such calculations in the anemias. Am. J. Med. Sciences, vol. 177, p. 513.

YOUMANS, J. B. 1941 Observations on nutrition in France. Trans. and Studies College of Physicians of Philadelphia, vol. 9, p. 144.

# THE EFFECTS OF A DIET DEFICIENT IN PART OF THE VITAMIN B COMPLEX UPON MEN DOING MANUAL LABOR

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There is urgent need for exact knowledge of the dietary needs of persons engaged in hard physical work. Surveys have shown that many manual laborers are living on diets that may be deficient in one or more essentials. Even more important at present, troops living on emergency rations, and sailors separated from bases, run the danger of having to work hard on diets that may be inadequate.

Optimal nutrition is necessary for securing the highest degree of physical fitness. Merely preventing deficiencies will not suffice. Most of the available data is concerned chiefly with either the appearance of clinical evidence of deficiency after a prolonged poor diet, or the earliest manifestations of deficiency in sedentary subjects or hospital patients. Very little is known about what happens when subjects, previously on a good diet, do heavy labor on a deficient diet. Specific practical questions are: (1) How soon does physical deterioration begin? (2) How much deterioration will occur in 1 week, the maximal period in which military emergency rations are expected to be used? (3) What are the conditions of recovery from such deficiency?

In the present experiments, ten men were subjected to heavy labor on a diet deficient in certain members of the vitamin B complex, but adequate in all other respects. Laboratory and field tests were performed to detect physical deterioration. Observations were made on the protective action of daily doses of 2 mg. of thiamine hydrochloride, and on the curative action of whole dried brewers' yeast.

## EXPERIMENTAL PROCEDURES

The experiments were planned as follows:

*Time table.* January 7–15, preliminary measurements made, and normal diet used. January 15–22, manual labor performed while eating a deficient diet. January 23–27, manual labor performed; same basal diet as before but with added daily dose of yeast. The manual labor consisted in chopping, sawing and splitting oak logs; in building breakwaters with large stones; and in walking. The shortest hike was $7\frac{1}{2}$ miles, the longest, 20. The daily requirement was between 4000 and 5000 calories.

*Subjects.* The subjects were ten healthy men, ranging from 23 to 40 years of age. It is important to note that the initial state of physical training was widely different from subject to subject, and also their relative physical endowments.

*Diet.* The diet is described in table 1. From previous experience with sedentary subjects it was known to be adequate (a) for maintenance of weight for 6 weeks; (b) in protein with respect to quantity (65 to 80 gm. per day) and in quality (cheese and egg white being a source of first-class protein); and (c) in vitamins A, C, and D. Data on the various members of the B complex in foods are incomplete, and the maintenance doses are not exactly known. However, by present standards (National Research Council, '41) the diet was certainly deficient in thiamine, probably deficient in nicotinic acid, pyridoxine and pantothenic acid, and probably adequate in riboflavin.

All of the subjects made their selections from the same group of foods, and the caloric intake was not restricted.

Capsules containing 2 mg. of thiamine hydrochloride were administered to five subjects every day at lunch, and at the same time the five others received placebos. The subjects did not know whether they received thiamine or placebos.

After 1 week of the deficient diet, the subjects began to take, instead of capsules, 18 gm. of whole dried brewers' yeast.[1] They ate the same deficient diet and continued to perform manual labor as before.

### TABLE 1

*Diet*

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**Foods allowed in unlimited amounts at any meal**

Cheese, rice, macaroni, spaghetti, soda crackers, butter, puffed rice (unfortified), honey, salad oil, shortening (lard or vegetable), sugar, coffee, tea, heavy cream, salt, vinegar, egg white (no yolk), baking powder biscuits (prepared from thiamine-free flour, no milk or yeast), tapioca (lemon flavor), hard candy, special cookies (made from thiamine-free flour, brown sugar, cream and water), and gelatine.

**Foods allowed in small servings and with special restrictions**

Either grape juice (small glass) or cranberry juice (small glass) once a day. Onions in small amounts were used at any time for flavoring.

Of the following, two and only two, were served each day: Beets (canned), spinach (boiled), lettuce (fresh), strawberries (frozen or canned), raspberries (frozen or canned), apple (one only, small), applesauce, banana (fresh), canned peaches.

Foods not specifically mentioned above were forbidden.

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The yeast contained the following vitamins, in micrograms per gram, the methods used being given in parentheses: thiamine (Schultz, Atkin and Frey, '42) 20–30; riboflavin (Kemmerer, '40) 70; nicotinic acid (Melnick and Field, '40) 600; pyridoxine (Williams, Eakin and McMahon, '41) 85; pantothenic acid (Pennington et al., '41) 200. The total protein content was 45%.

It will be seen that the thiamine content of this yeast was relatively low, so that the subjects who had previously received 2 mg. of thiamine hydrochloride per day now received only ½ mg. per day.

[1] This yeast (type 2019) was donated by Standard Brands, Inc.

For purposes of clarity the subjects who received placebos will be called the "deficient" subjects, and those who received, during the first half of the experiment, 2 mg. of thiamine hydrochloride per day will be called the "thiamine" subjects. The first half of the experiment will be called the "deficient" period, since even the "thiamine" subjects failed to receive certain other needed vitamins of the B complex; the second half will be called the "yeast" period, since all subjects were then taking yeast every day.

*Field measurements of physical fitness.* Each day, and sometimes morning and evening, every subject's physical condition was tested quantitatively by a test devised in the Harvard Fatigue Laboratory for use in the field. It consists of pulling a stoneboat loaded so that the pull is $\frac{1}{2}$ the subject's body weight, over a flat course 300 yards in length, at a rate of 1 yard per second. A subject may be forced to stop exhausted before 300 yards. The score is calculated from the formula:

$$\text{Fitness index} = \frac{\text{Duration of standard exhausting exercise} \times 100}{2 \times \text{sum of pulses taken from 1 to } 1\frac{1}{2}, 2 \text{ to } 2\frac{1}{2}, 4 \text{ to } 4\frac{1}{2} \text{ minutes of recovery}}$$

(Johnson, Brouha and Darling, '42).

A good score is above 80, a poor one below 40. This test is useful both in evaluating the physical fitness of subjects for exhausting exercise, and in following the state of training of any individual subject. Each evening private interviews were held with each subject to review the symptoms of the day.

*Laboratory measurements of physical fitness.* More extensive measurements of physical fitness were made on each subject on three occasions: (a) Before starting the deficient diet and the heavy work; (b) 1 week after starting the deficient diet and manual labor; (c) 5 days after beginning to take yeast along with the deficient diet and continued manual labor. The tests were carried out as follows: The subject ran on a motor-driven treadmill for 15 minutes on the level at a speed of 5.8 m.p.h., 7.0 m.p.h., or 8.6 m.p.h., depending on his physical condition. During this run the pulse rate was re-

corded continuously by means of a cardiotachometer, and ventilation, oxygen consumption, and carbon dioxide output were measured. After resting 6 minutes the subject ran at the same speed as before but at a grade of 8.6%, until he could run no longer. The heart rate was recorded continuously during the run and for 6 minutes afterwards.

After resting ½ hour, the subject again ran to exhaustion at the same grade and speed. His pulse was recorded during recovery at intervals for 6 minutes.

*Other observations.* Before the subjects left for the island where the experiments took place, 24-hour samples of urine were collected for estimation of thiamine; others were collected after the subjects had been on the diet 6 and 7 days. Electrocardiograms were taken and heart size estimated by x-ray in the normal, in the deficient, and, in some cases, in the yeast periods.

### RESULTS

*Caloric value of the diet.* The requirement of the subjects was calculated to be from 4000 to 5000 calories a day. The caloric value of the diet was adequate since all subjects went through both the deficient and the yeast periods without significant change in weight. The maximal change of any subject's weight was 1.5 kg., and the average net weight change was —0.3 kg.

*Urinary findings.* Thiamine in the urine was estimated by the method of Egaña and Meiklejohn ('41). At the end of the deficient period, the five "deficient subjects" were excreting thiamine in amounts well within the range usually taken as indicative of deficiency (Robinson, Melnick and Field, '40); the average excretion was 29 µg. of thiamine in 24 hours. The five "thiamine subjects" excreted amounts of thiamine well up in the normal range; the average was 200 µg. in 24 hours. The urinary findings prove conclusively that the "deficient subjects" depleted their stores of thiamine significantly within 7 days.

*Symptoms.* The "deficient subjects" all developed within 6 days a majority of the following symptoms, sometimes

acutely: (a) A general feeling of lassitude, inefficiency and depression. (b) Easy fatigue, especially in climbing stairs and hills. (c) Muscle and joint pains different from the stiffness following unaccustomed exercise: instead of disappearing after a few days of exercise, they grew steadily worse. The pains came abruptly after motion was started, but did not diminish during the day's work and stopped a few hours after work was over. (d) Poor appetite and even antipathy to food, even though the subjects forced themselves to eat enough to maintain weight. (e) Irregular bowel habits taking the form of severe constipation or scanty, hard stools. (f) "Slowness" was a vague but prominent symptom, the subjects being unable to accomplish fast motion when they wished. (g) Additional psychic changes in the form of quarrelsomeness and irritability appeared in two subjects.

In contrast to the "deficient subjects," the "thiamine subjects" had few symptoms, and these were mild. However, one complaint common among them was easy fatigue.

Following the administration of yeast, there was a lag of 48 hours before the subjects began to feel better. Then the improvement in feeling occurred very suddenly, and could be timed almost to the hour. By the end of 5 days no subject had any complaints although the same diet (with the addition of yeast only) was still being eaten.

*Results of measurements of physical fitness in the field.* The results of the daily measurements of fitness are shown in figure 1. It will be seen that both groups of subjects showed marked deterioration in physical state within 6 days, but those receiving thiamine showed this in somewhat less degree. After yeast was administered, all subjects recovered, the thiamine subjects quicker and more completely than the others.

*Results of measurements of physical fitness on the treadmill.* In general, for moderate exercise (i.e., running on the level) the "deficient subjects" showed, in the deficient period, abnormally high pulmonary ventilation and moderately increased oxygen consumption, whereas the "thiamine subjects" altered only slightly in these respects. Ventilation was increased especially in those subjects who had complained most

EFFECT OF DEFICIENT DIET ON PHYSICAL FITNESS
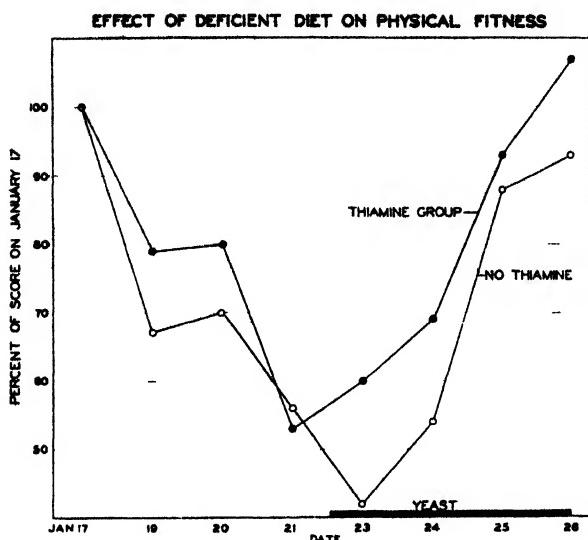
Deterioration in physical fitness for hard work following subsistence on a diet deficient in the vitamin B complex, and improvement following daily addition of whole brewers' yeast to the deficient diet, starting on January 22. Open circles: Average for "deficient subjects." Dots: Average for "thiamine subjects."

of easy fatigue. For instance, the ventilation of the deficient subject *We* was 890, 1062 and 869 cc. per kilo per minute in the control, deficient and yeast periods, respectively, and the corresponding oxygen consumption was 46.5, 48.3 and 47.5 cc. per kilo per minute. This phenomenon is comparable to what has been seen in sedentary subjects submitted to a vitamin B deficient diet for at least 4 weeks.[2] These findings indicate an impaired mechanical efficiency [3] for moderate work.

[2] Recent observations from these Laboratories (Egaña et. al., '42).

[3] The term "mechanical efficiency" is used in this paper in its strict sense, i.e., the ratio of effective external work performed to the total caloric output during work that is maintained in a steady state. The tests of physical fitness used on our subjects are designed to test endurance for long sustained exertion, such as walking 40 miles (Johnson, Brouha and Darling, '42). Recuperation is measured by repetition of the standard exhausting exercise. The performance of the second bout of exhausting exercise is an indication of the effectiveness of the recovery processes which enable a man to carry out repeated exhausting tasks. This type of recovery, when effective, enables some athletes to run both the mile race and the 2-mile race on a single afternoon. It is of the greatest importance to manual laborers, if they are to work effectively.

Two conclusions can be drawn from the performance of exhausting exercise, i.e., running uphill (table 2): (1) Both groups of subjects exhibited marked deterioration during the deficient period, especially in the capacity to perform repeated exhausting exercise. This is directly opposite to what was seen in two of the same group of men in a later experiment, when they did daily manual labor while subsisting on a normal diet. (2) Both groups of subjects improved strikingly during the yeast period. In fact, as a result of training, seven became fitter than they had been when eating a normal diet.

TABLE 2

*Response of two individual subjects to repeated exhausting exercise at 7.0 m.p.h. and 8.6% grade.*

*Results are expressed as fitness indices (Johnson, Brouha and Darling, 1942).*

| | DEFICIENT SUBJECT HOL | | THIAMINE SUBJECT JOH | |
|---|---|---|---|---|
| | 1st run score | 2nd run score (½ hr. later) | 1st run score | 2nd run score (½ hr. later) |
| A. Period of normal diet | 133 | 141 | 83 | 86 |
| B. "Deficient" period | 122 | 105 | 63 | 67 |
| C. "Yeast" period | 138 | 107 | 85 | 95 |

*Cardiological observations.* No significant changes were observed in the electrocardiogram of most subjects. In two instances slight lowering of the T waves was observed and may have been due to the vitamin deficiency.[4] Because of the small magnitude of the changes, the electrocardiogram is of little help in detecting early stages of this type of vitamin deficiency. However, a significant increase in the amplitude of T following the administration of vitamin B complex would strongly suggest that a state of deficiency existed previously.

Teleroentgenograms of the chest taken before and at the end of the deficient period showed no significant difference in heart size.

[4] The "deficient subject" WY developed cheilosis of marked degree, and a sore tongue, at which time the electrocardiogram showed nodal rhythm with significantly lower T waves in Leads I, II, and CF4.

## DISCUSSION

The practical conclusion of these experiments is that any person engaging in daily hard physical work needs imperatively an adequate intake of the vitamin B complex every day if he is to remain fit. Brewers' yeast was a complete and adequate supplement to the deficient diet used. It abolished in every subject the symptoms and signs of deficiency. In the diets of workers, natural food products can supply the necessary daily vitamin intake in a palatable form and without risk of deficiency. If extra vitamins are ever indicated for any ration, addition of brewers' yeast at present seems a sure way of avoiding deficiency in any single component of the B complex.

Certain observations deserve special emphasis: (1) Our subjects developed symptoms and definite signs of deterioration far more quickly and more severely than sedentary subjects on a similar diet (e.g., the subjects of Williams, Mason, Wilder and Smith, '40). (2) Recovery after deficiency was a relatively slow process, and three of the "deficient subjects" were not yet completely normal after taking yeast for 5 days. (3) The abnormally increased ventilation and oxygen consumption during moderate work which was especially marked in three deficient subjects would be a great disadvantage where air and oxygen are at a premium, e.g., at high altitudes and in diving operations. (4) Impairment of the ability to carry out repeated exhausting tasks is a great disadvantage to anyone engaged in sustained physical exertion.

It is worth emphasizing that marked physical deterioration occurred in spite of the ingestion of 2 mg. of thiamine hydrochloride per day which clearly prevented the thiamine group from experiencing the muscle pains and other symptoms reported by the "deficient subjects." When yeast, containing only $\frac{1}{2}$ mg. of thiamine per day, was administered, physical improvement occurred. It seems possible from these results to distinguish some of the characteristics of both early thiamine deficiency and early deficiency with respect to other parts of the vitamin B complex. The "deficient subjects" had

marked symptoms, the "thiamine subjects," few. Thiamine deficiency would therefore seem to cause symptoms. Both groups of subjects developed marked signs of deterioration, the "deficient subjects" more than the others. This moderate extra deterioration would seem to be caused by thiamine deficiency in addition to the other deficiencies. Presumably, the thiamine hydrochloride did not prevent the deterioration which was cured by the yeast. This conclusion is strengthened by the fact that both groups recovered their physical fitness while taking yeast which contained thiamine in doses only one-quarter as large as the "thiamine subjects" had previously received.

For these reasons, in working men, we consider early thiamine deficiency to be characterized by marked symptoms and moderate physical deterioration, and early deficiency in other members of the vitamin B complex to be characterized by few symptoms but marked physical deterioration.

## SUMMARY

Ten men were subjected to hard daily physical work on a diet deficient in parts of the B complex, notably in thiamine, and adequate in all other respects. During the first week of the experiment five subjects received 2 mg. of thiamine hydrochloride per day, the other five, placebos. During the last week of the experiment all subjects received daily doses of 18 gm. of brewers' yeast containing ½ mg. of thiamine.

The main results were as follows: (a) At the end of 1 week all subjects complained of easy fatigue and their physical fitness had deteriorated markedly; this was greater in the subjects without thiamine. (b) A majority of subjects without thiamine exhibited symptoms, sometimes acute, of muscle and joint pains, lack of well-being, poor appetite and constipation. These symptoms were mild or absent in the subjects receiving thiamine. (c) During the "yeast period" all symptoms disappeared and the usual level of fitness was regained more rapidly and more completely by the subjects who had received thiamine during the first week. (d) Changes in the electro-

cardiograms of certain subjects in both groups that were noticed at the end of the deficient period had disappeared by the end of the yeast period. (e) At the end of the first week, the daily urinary excretion of thiamine by the subjects who had not received this vitamin was low and well within the range characteristic of deficiency.

The following conclusions are drawn: (a) When men are doing hard physical work even for a few days, there is an imperative need for an adequate daily intake of the vitamin B complex if physical fitness is to be maintained. (b) Of the vitamin B complex thiamine alone will not maintain the physical fitness of laborers in single daily doses of 2 mg. (c) Whole dried brewers' yeast, on the other hand, is a complete and adequate supplement for a diet grossly deficient in the vitamin B complex. (d) When addition of vitamin B complex to a ration is indicated, a natural product such as yeast would seem to be a sure source of all the necessary components.

### LITERATURE CITED

EGAÑA, E., R. E. JOHNSON, R. BLOOMFIELD, L. BROUHA, A. P. MEIKLEJOHN, J. WHITTENBERGER, R. C. DARLING, C. HEATH, A. GRAYBIEL AND F. CONSOLAZIO 1942 The effects of a diet deficient in the vitamin B complex on sedentary men. Am. J. Physiol., vol. 137, p. 731.

EGAÑA, E., AND A. P. MEIKLEJOHN 1941 The estimation of thiamine in urine. J. Biol. Chem., vol. 141, p. 859.

JOHNSON, R. E., L. BROUHA AND R. C. DARLING 1942 A test of physical fitness for strenuous exertion. Revue Canadienne de Biologie, vol. 1, p. 491.

KEMMERER, A. R. 1940 Report on riboflavin (assay). J. Assoc. Off. Agric. Chem., vol. 23, p. 346.

MELNICK, D., AND H. FIELD, JR.   1940   Chemical determination of nicotinic acid: Inhibitory effect of cyanogen bromide upon the aniline side reactions. J. Biol. Chem., vol. 135, p. 53.

NATIONAL RESEARCH COUNCIL, COMMITTEE ON FOOD AND NUTRITION   1941   Recommended Dietary Allowance, Washington, D. C.

PENNINGTON, D. E., E. E. SNELL, H. L. MITCHELL, J. R. McMAHON AND R. J. WILLIAMS   1941   Assay method for pantothenic acid. Univ. Texas Publication No. 4137, p. 14. Univ. of Texas, Austin.

ROBINSON, W. D., D. MELNICK AND H. FIELD, JR.   1940   Urinary excretion of thiamine in clinical cases and the value of such analyses in the diagnosis of thiamine deficiency. J. Clin. Invest., vol. 19, p. 399.

SCHULTZ, A. S., L. ATKIN AND C. N. FREY   1942   Determination of vitamin $B_1$ by yeast fermentation method. Improvements related to use of sulfite cleavage and a new fermentometer. Ind. & Eng. Chem. (Anal. Ed.), vol. 14, p. 35.

WILLIAMS, R. J., R. E. EAKIN AND J. R. McMAHON   1941   Assay method for pyridoxin. Univ. Texas Publication No. 4137, p. 24. Univ. of Texas, Austin.

WILLIAMS, R. D., H. L. MASON, R. M. WILDER AND B. F. SMITH   1940   Observations on induced thiamine (vitamin $B_1$) deficiency in man. Arch. Int. Med., vol. 66, p. 785.

# RELATIONSHIP BETWEEN VITAMIN A AND IODINE METABOLISM IN THE RAT [1,2]

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In 1936 Remington and Levine showed that in rats maintained on an iodine deficient regime, certain quantitative relationships exist in the weight, iodine content, and dry matter content of the thyroid glands, and between each of these and the iodine supply. These criteria, together with histological examinations, have since been used in studying the effect upon the gland of superposing dietary or other deficiencies or abnormalities upon the iodine deficiency (Remington, J. W., '37; Remington, R. E., '38 a, '38 b; Harris and Remington, '39) with the hope of adding to our knowledge of the specific relationships of the thyroid in general metabolism, as well as to reveal contributory or secondary causes of endemic goiter if such exist.

The primary object in undertaking the experiments to be reported herein was to determine whether lack of vitamin A could enhance the severity of goiter due to an iodine deficiency. However, considerable work has been done and evidence accumulated bearing on other phases of the thyroid-vitamin A relationship, and our experimental approach has therefore been extended to include such questions as the effects of goiter, hypo- and hyperthyroidism on carotene utilization and vitamin A metabolism and requirement.

According to McCarrison ('30) the thyroid is unable to deal with iodine in a normal manner in the absence of a sufficiency of vitamin A, and goiter results. Supporting evidence is that of Mitzkewitsch ('34) who reported that in rats which died of avitaminosis A the thyroids were enlarged but their microscopic appearance indicated hypofunction, while de Ruyter ('34) found atrophy of the thyroid in severe vitamin A deficiency. In neither case is there reported any control over the iodine intake of the animals, but similar results have been reported by Coplan and Sampson ('35), the iodine content of whose diets was known. On the other hand Sherwood and Luckner ('35) observed hyperplastic thyroids, deficient in colloid, in rats fed large doses of vitamin A in the form of cod liver oil, halibut liver oil, or carotene.

Another phase of the thyroid-vitamin A relationship that has received attention is the effect of vitamin A on hyperthyroidism. Abelin ('35) has shown that the toxic effects of ingested thyroid substance or injected thyroxine can, in a measure, be offset by vitamin A. According to Logaras and Drummond ('38) such an effect can be observed with massive doses of vitamin A, but is negligible or absent when the vitamin A intake is sufficient only to supply the needs of the animal. Clinically vitamin A has been found of value in relieving symptoms of toxic goiter (Wendt, '35 a; Dietrich, '36), and Abelin ('36) recommends doses of 150,000 rat units per day in the treatment of hyperthyroidism.

There is also some evidence bearing on effects of thyroid activity on vitamin A and carotene metabolism. Wendt ('35 b) reported impaired ability to convert carotene into vitamin A in myxedema and cretinism, and Fasold and Heidemann ('33) found that after thyroidectomy goats secreted milk containing carotene but no vitamin A, whereas normally the reverse was true. This they explained by saying that the lowered general metabolism after thyroid removal limits the ability to transform carotene to vitamin A. The time required to deplete rats of vitamin A is markedly prolonged by thyroidectomy, but shortened when intact animals are injected with thyroxine

(Greaves and Schmidt, '36). The administration of carotene protects against loss of weight in rats injected with thyroxine (Euler and Klussmann, '32); and thyroxine protects against the harmful effects of massive doses of vogan (a vitamin A concentrate) according to Fasold and Peters ('33). Clausen and McCoord ('38) found that in fevers, including artificial hyperthermia, there is a drop in both the carotene and vitamin A content of human blood. This has been confirmed by Thiele and Scherff ('39 a, b) who also reported that these values are always low in Graves' disease, but not in colloid goiter.

## EXPERIMENTAL

*Deficiency of both vitamin A and iodine: Effect on thyroid of feeding vitamin A and carotene with and without iodine*

Inspection of a number of diets previously investigated as to goiter production and growth (Remington, R. E., '37) revealed that a diet of wheat gluten, yellow corn meal, dried brewers' yeast, calcium carbonate and salt would prove satisfactory in producing a degree of goiter which would lie on a critical part of the curve, and could also be made deficient in vitamin A by replacing the yellow corn meal with sucrose. It seemed desirable, furthermore, when using so highly purified a diet, to add a more complete salt mixture, for which purpose mixture 351 of Hubbell, Mendel and Wakeman ('37) was made up without potassium iodide. The diet selected consisted of sucrose 70 parts, wheat gluten 18, dried brewers' yeast 5, cottonseed oil (containing enough viosterol to yield 3 U.S.P. units of vitamin D per gram of diet) 5, and salt mixture 2 parts. The first experiment was designed to compare the degree of goiter existing in rats equally deprived of iodine, but receiving graded levels of vitamin A as such or as carotene. The source of vitamin A was a concentrate prepared from fish liver oil by molecular distillation,[4] and the carotene a commercial product [5] stated to contain 90% beta-

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[4] Distillation Products, Inc., Rochester, N. Y.
[5] S M A Corporation, Cleveland, Ohio.

and 10% alpha-carotene. Weanling rats were placed on the
diet described above and maintained without addition of any
supplement until depleted of vitamin A, as evidenced by a
cessation of growth for 7 days, at which time they were divided
into groups of eight animals each, and fed either vitamin A

TABLE 1

*Effect on the thyroid gland of feeding vitamin A and carotene, with and without
iodine supplementation, to rats previously depleted as to both vitamin A and iodine.*

| SUPPLEMENT U.S.P. UNITS PER WEEK | WEIGHT GAIN IN 5 WEEKS | THYROID GLANDS | | |
|---|---|---|---|---|
| | | Weight per 100 gm. body wt. | Dry matter | Iodine dry basis |
| A. Diet deficient in both vitamin A and iodine | | | | |
| | *gm.* | *mg.* | *%* | *%* |
| none | —1.5 | 23.4 | 22.8 | .020 |
| | (in 2 weeks) | | | |
| 6 vitamin A | 7.9 | 27.8 | 23.1 | .012 |
| 60 vitamin A | 13.8 | 26.2 | 21.8 | .018 |
| 600 vitamin A | 56.0 | 27.2 | 25.0 | .019 |
| 6 carotene | 8.8 | 31.6 | 22.8 | .018 |
| 60 carotene | 16.4 | 24.7 | 24.3 | .025 |
| 600 carotene | 62.4 | 29.0 | 21.7 | .020 |
| B. Diet deficient in vitamin A but supplying about 2 μg. per day of iodine | | | | |
| none | | 13.9 | 28.5 | .180 |
| 6 vitamin A | | 14.3 | 26.9 | .204 |
| 60 vitamin A | | 13.0 | 27.7 | .192 |
| 6 carotene | | 15.7 | 30.4 | .200 |
| 60 carotene | | 15.2 | 29.0 | .220 |
| Colony ration (fully adequate) | | 10.0 | 33.3 | .200 |

or carotene at levels of 3, 30, and 300 U.S.P. units twice weekly
for 6 weeks, rats of the control group meanwhile receiving
just enough vitamin A to keep them alive. The results [6] are
shown in the first part of table 1. From previous experience
with diets of this type, that degree of thyroid hyperplasia
which any given diet is capable of producing will be attained

[6] The glands from each group were assembled in a small nickel boat and, after
drying, handled essentially as described by Remington et al. ('30), except that
the final estimation was made by a micro-modification of the thiosulphate titration.

in 5 weeks or less, after which the thyroid values remain rather constant. Since the average time required for depletion in vitamin A was more than 5 weeks, what was being observed here was the ability of vitamin A and carotene to render more efficient the utilization of the unavoidable minimum of iodine in the diet for the relief of a previously existing thyroid hypertrophy. It will be seen from the data that no significant differences are apparent in the constants on the thyroid glands of the different groups, i.e., vitamin A and carotene at the levels fed are not able to render more efficient the use of iodine in such minute amounts by hyperplastic glands.

It may be that in the above experiment the iodine restriction is so severe as to nullify any possible effect of the vitamin, in which case it would be more logical to perform the experiment on rats receiving nearly, but not quite, a fully protective daily dose of iodine. The experiment was repeated, with the difference that sodium iodide was added to the diet in an amount that would provide an average daily intake of 2 µg. iodine per rat, this being slightly less than the 3 µg. which we consider the minimum adequate level for normal thyroid composition and structure. Here again (table 1, part B) although the thyroid constants are quite different from those of the former experiment, and more nearly like those of normal animals (Remington, Remington, and Welch, '37), there is no definite indication that the addition of vitamin A or carotene has affected either the degree of enlargement or the utilization of iodine by the thyroid.

*Deficiency of vitamin A: Effect of administering iodide and desiccated thyroid on eye symptoms and survival*

Since vitamin A is apparently without effect upon the development of low iodine goiter under the conditions of the preceding experiment, a study was next undertaken to determine whether the converse is true, i.e., whether a degree of goiter, or a deficiency in the thyroid hormone itself, would have any effect on the requirement for or utilization of vitamin A. Six groups of weanling rats were placed on the same

diet as before, with various supplements as indicated, from
the beginning, as shown in table 2. Animals of the control
group, receiving only the basal diet, developed xerophthalmia
after an average period of 51 days, and died within 6 or 7
days with typical post-mortem symptoms of avitaminosis A
as well as enlarged and hyperplastic thyroids. Addition of

TABLE 2

*Effect of administering iodide and desiccated thyroid gland, on the incidence
of eye symptoms and the survival period of normal and thyroidectomized
rats deprived of vitamin A.*

| NUMBER OF ANIMALS | TREATMENT | SUPPLEMENT (FED DAILY) | APPEARANCE OF EYE SYMPTOMS | SURVIVAL PERIOD | REMARKS |
|---|---|---|---|---|---|
| | | | *(days)* | *(days)* | |
| 10 | ... | ..... | 51 | 58 | Thyroids definitely hyperplastic |
| 10 | ... | 5 µg. iodine | 49 | 60 | Thyroids normal |
| 10 | ... | 20 mg. thyroid per 100 gm. body weight | 28 | 35 | Thyroids normal |
| 5 | | 20 mg. thyroid per 100 gm. body weight plus 5 µg. iodine | 30 | 35 | Thyroids normal |
| 8 | Thyroids removed | 20 mg. thyroid per 100 gm. body weight | 55 | 61 | At autopsy gross and microscopic examination failed to reveal any vestiges of remaining thyroid tissue |
| 8 | Thyroids removed | ..... | 83 | 98 | |

5 µg. per day of iodine as sodium iodide did not affect the
vitamin A depletion time or the survival time, but the thyroids
were grossly and histologically normal. A third group re-
ceived daily doses of commercial desiccated thyroid gland in
an amount which, in animals of our stock colony, would result
in slightly decreased growth. Here the onset of xerophthalmia
occurred in 28 days, death following in 7 days more; this is

a marked shortening of the vitamin A depletion time which we, in the absence of more specific indications, are inclined to attribute to the heightened general metabolism due to the desiccated thyroid. The thyroid glands were normal, having been spared the effects of the iodine deficient diet by the desiccated thyroid. This picture is not affected when 5 µg. per day of iodine is given with the desiccated thyroid. Two additional groups were thyroidectomized at 28 days of age, 6 days after being placed on the experimental diet. Of these, one group received desiccated thyroid, the other no supplement. In the thyroid-fed group the vitamin A depletion time and survival period were substantially the same as those of unoperated animals without dietary supplement, but the thyroidectomized group without supplement, while not making much gain in weight after the operation, continued in apparent good condition for an average of 83 days before symptoms of vitamin A deficiency appeared; they survived for 15 days longer, dying with typical symptoms of avitaminosis A. These observations confirm the findings of Greaves and Schmidt. However, not being able to reproduce the effect of excess thyroxine with dinitrophenol, they did not believe that the change in basal metabolic rate was responsible for the change in the rate of vitamin A depletion. On the other hand, the finding of Clausen and McCoord that plasma vitamin A values are lowered in fevers and artificial fevers, as well as in toxic goiter, would imply that there is an increased need for vitamin A which parallels, at least, the general heat production. All of the thyroidectomized rats were not examined at autopsy for histological evidence of complete thyroid removal, but seven representative animals were so examined and no evidence of functional thyroid tissue found.[7] Comparing the depletion time of the thyroidectomized rats with that of intact animals, with and without iodine, reveals that a degree of iodine deficiency which brings about a decrease of 90% in the iodine storage of the thyroid, with a corresponding

decrease in colloid and other characteristic changes, has no effect on the vitamin A depletion time, whereas complete removal of the thyroid does. It must be that even so severe a degree of goiter as is represented in these experiments has an insignificant effect on the general metabolism as compared with complete extirpation of the gland. In confirmation of the above, one of us (R. E. R.) has determined the basal metabolic rate of a considerable number of iodine-deficient rats over a period of several years, with the conclusion that there is only a slight and erratic tendency toward a rate lower than that of normal animals.

One other postulation can be tested by this technique, i.e., that hypofunction of the thyroid decreases the ability of the organism to convert dietary carotene into vitamin A. In the first experiment we observed that gain in weight, when equal supplements of vitamin A and carotene were fed to depleted, goitrous rats, was practically the same for either form, thus indicating complete conversion. However, it is only an assumption that the goiter was severe enough to affect the functional state of the gland as related to general metabolism. Hence, a number of rats were thyroidectomized and maintained on the basal diet until weight was constant or declining for 7 days, when they were divided into groups and given, respectively, 1, 3, and 30 U.S.P. units per day of vitamin A or carotene. Rather rapid alleviation of eye symptoms occurred at the highest level of either vitamin source. When 1 unit of either source was fed the cure required 7 to 9 days; with 3 units, 4 days were sufficient for either source. Even at the low critical level of 1 unit per day there was no evidence that carotene was any less efficient than vitamin A in relieving xerophthalmia. In this experiment the animals were considered as being depleted of their vitamin A stores 7 days after the first appearance of a bloody exudate from the eyes, regardless of weight. Cure of xerophthalmia was considered to have occurred when the eyes had returned to as near normal as possible. Experience in conducting vitamin A bioassays enables one to judge this "cure" with considerable consistency.

Due to these rats having been thyroidectomized, there was little or no stimulation of growth at any level of vitamin A supplementation.

## CONCLUSIONS

It may be concluded from these experiments, that in rats:

1. There is no reason to believe that vitamin A deficiency is an etiological factor in the development of simple goiter.

2. There is apparently an increased need for vitamin A in hyperfunction of the thyroid, probably corresponding to the increase in the rate of general metabolism, and a correspondingly decreased need in thyroidectomized animals; but in a degree of goiter in which the iodine storage of the thyroid is decreased by 90%, the latter effect is not demonstrated.

3. A deficiency of the thyroid hormone does not adversely affect the ability of the animal to utilize carotene in the cure of xerophthalmia, as compared with pre-formed vitamin A.

## LITERATURE CITED

ABELIN, I. 1935 Ueber den Antagonismus A-Vitamin: Thyroxin. Ztschr. f. Vitaminforsch., vol. 4, p. 120.

———— 1936 Ueber die medikamentöse Anwendung des A-Vitamins bei der Hyperthyreose. Schweiz. med. Wchschr., vol. 66, p. 1106.

CLAUSEN, S. W., AND A. B. McCOORD 1938 Carotenoids and vitamin A in the blood. J. Pediat., vol. 13, p. 635.

COPLAN, H. M., AND M. M. SAMPSON 1935 The effects of a deficiency of iodine and vitamin A on the thyroid gland of the albino rat. J. Nutrition, vol. 9, p. 469.

DIETRICH, H. E. 1936 Vitaminbehandlung dur Schilddrüsenüberfunktion mit Vogan. München. med. Wchschr., vol. 83, p. 313.

EULER, H. V., AND E. KLUSSMANN 1932 Carotin (Vitamin A) und Thyroxin. Ztschr. f. physiol. Chem., vol. 213, p. 21.

FASOLD, H., AND E. R. HEIDEMANN 1933 Ueber die Gelbfärbung der Milch thyreopriver Ziegen. Ztschr. f. d. ges. exper. Med., vol. 92, p. 53.

FASOLD, H., AND H. PETERS 1933 Ueber den Antagonismus zwischen Thyroxin und Vitamin A. Ztschr. f. d. ges. exper. Med., vol. 92, p. 57.

GREAVES, J. D., AND C. L. A. SCHMIDT 1936 Studies on the vitamin A requirement of the rat. Am. J. Physiol., vol. 116, p. 456.

HARRIS, P. L., AND R. E. REMINGTON 1939 The effect of yeast and of thiamin on the production of low iodine goiter. J. Nutrition, vol. 17, p. 31.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, p. 273.

LOGARAS, G., AND J. C. DRUMMOND 1938 Vitamin A and the Thyroid. Biochem. J., vol. 32, p. 964.

MCCARRISON, R. 1930 Further researches on lymph-adenoid goitre in rats. Indian J. Med. Research, vol. 18, p. 577.

MITZKEWITSCH, M. S. 1934 Der Zustand der endokrinen Drüsen während der A-Avitaminose bei Ratten. Arch. f. exper. Path. u. Pharmakol., vol. 174, p. 339.

REMINGTON, J. W. 1937 Low iodine goiter and the resistance of the rat to thyreotropic stimulation. Proc. Soc. Exp. Biol. Med., vol. 37, p. 175.

REMINGTON, R. E. 1937 Improved growth in rats on iodine deficient diets. J. Nutrition, vol. 13, p. 223.

———— 1938 a Does fat in the diet affect the thyroid? J. Nutrition, vol. 16, p. 417.

———— 1938 b The effect of chloride on the thyroid gland. Proc. Soc. Exp. Biol. Med., vol. 37, p. 652.

REMINGTON, R. E., AND H. LEVINE 1936 Studies on the relation of diet to goiter. III. Further observations on a goitrogenic diet. J. Nutrition, vol. 11, p. 343.

REMINGTON, R. E., J. F. MCCLENDON, HARRY VON KOLNITZ AND F. B. CULP 1930 The determination of traces of iodine. IV. Iodine in small quantities of thyroid and other tissues. J. Am. Chem. Soc., vol. 52, p. 980.

REMINGTON, R. E., J. W. REMINGTON AND S. S. WELCH 1937 The thyroid gland of the normal rat: Size, dry matter and iodine content. Anatomical Record, vol. 67, p. 367.

DE RUYTER, TH. H. 1934 Investigations into the histology of the A-avitaminosis. I. Alterations in organ and tissue structure. Acta brev. Neerland., vol. 4, p. 122.

SHERWOOD, T. G., AND W. G. LUCKNER 1935 Further studies on the effect of cod liver oil on the thyroid gland. J. Nutrition, vol. 9, p. 123.

THIELE, W., AND I. SCHERFF 1939 a Über die pathogenetische und diagnostische Bedeutung des Carotin- und Vitamin A- Spiegels im Serum. Klin. Wochenschr., vol. 18, p. 1208.

———— 1939 b Der Serum-Vitamin A-Spiegel in Fieber. Klin. Wochenschr., vol. 18, p. 1275.

WENDT, H. 1935 a Ueber die Behandlung der Basedow'schen Krankheit mit grossen Dosen Vitamin A (Vogan). München. med. Wchschr., vol. 82, p. 1160.

———— 1935 b Ueber Veränderungen im Karotin-Vitamin A-Haushalt beim Myxödem und bei Kretins. München. med. Wchschr., vol. 82, p. 1679.

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